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The effect of dietary coconut in tauopathies causing dementia

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ABSTRACT

Alzheimer's disease (AD) and Frontotemporal dementia (FTD) are the two most common forms of dementia. As tau neuropathology can be exhibited in both AD and FTD they belong to a group of neurodegenerative diseases called tauopathies, where underlying pathology is characterised by increased expression of abnormally hyperphosphorylated protein tau, its deposition and aggregation into neurofibrillary tangles. Despite numerous clinical trials and ongoing research, there are no curative, disease modifying or preventative treatments available for either AD or FTD at present, and their treatments are mainly limited to symptomatic management. Therefore, patients and caregivers have turned to adapting alternative approaches such as specialised diets. Coconut oil, being a naturally occurring ketogenic ingredient that can be easily and cost-effectively integrated into one's diet have been strongly promoted by the media. However, quality evidence from laboratory-based research or randomised, placebo-controlled clinical trials are lacking. In this study, the cognitive domains of spatial learning and memory were analysed using the Morris Water Maze (MWM) swim path strategies of TAU58/2 mice and wild-type controls who were fed specialised diets consisting of coconut-derived or lard-derived fat compared to control mice fed a standard chow diet. Immunofluorescent staining of post-mortem brain sections of these mice and statistical analysis of staining intensities were performed to determine the extent of tau neuropathology and neuroinflammation in the hippocampus. The results of the MWM swim path analysis revealed that transgenic mice fed with the coconut fat diet displayed significantly higher hippocampal-dependent cognitive behaviour (swim patterns) compared to the other two diet cohorts stipulating that incorporation coconut fat into one's diet may be beneficial in retaining learning and memory in AD and FTD. Although no difference between immunofluorescent staining intensities of antibodies against microglia (IBA1) and astrocytes (GFAP) was observed, the findings of this study indicate that there is a need to explore other avenues regarding neuroinflammation.

DECLARATION

The work presented in this thesis entitled "The effect of dietary coconut in tauopathies causing dementia" is the outcome of my own effort. Any ideas, data images or text resulting from work of others are fully identified as such; I have adequately cited and referenced the original sources. This thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university or institution other than Macquarie University.

The research presented in this thesis was approved by the Macquarie University Animal Ethics Committee, reference number:

Biosafety Approval:

0101- 520170101201

0335- 520170101201

Animal Ethics Approval:

AEC Reference No.: 2018/019

AEC Reference No.: 2017/053

(The two biosafety applications listed above contain my name. However, I am not named in the two animal ethics applications as my work did not include physically working with mice.

Liyana A. S. S. Kalupahana

8th December 2021

STATEMENT

Morris Water Maze (MWM) data were obtained previously in the DRC by Dr Annika van Hummel. All the video data obtained in the MWM trials were analysed using Anymaze video tracking software by me. I, Liyana Kalupahana and Dr Annika van Hummel (two independent researchers), each scored the swim paths twice, before a final score was discerned by me. The statistical analysis of the MWM swim paths, learning curve and probe trial data were done by me. All the sectioning and staining of the fixed mouse brain tissue for the histological analysis were done by me. Scanning of the stained tissue slides with the Axio scanner was done by Ms Sammi Knott, Research Assistant at DRC. Tracing of the stained hippocampi and quantification of staining via the Zen-blue software was done by me. All the statistical analyses in this thesis were done by me.

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I am immensely thankful to my family and friends for their continued encouragement, and to my husband Shavindra for his constant and sincere support with all my academic pursuits.

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AD: Alzheimer's disease

ALS: Amyotrophic lateral sclerosis

ApoE: Apolipoprotein E

APP: Amyloid Beta Precursor Protein

ATP: Adipose triphosphate

Aβ: Amyloid beta

CFD: Coconut-derived fat diet

CO: Coconut oil

DRC: Dementia Research Centre

FTD: Frontotemporal dementia

FTLD-tau: Frontotemporal lobar degeneration-tau pathology

LD: Lard diet

MAPT: Microtubule-associated protein Tau

MCI: Mild cognitive impairment

MIND diet: The Mediterranean diet and the Dietary Approaches to Stop Hypertension: DASH diet

MND: Motor neuron disease

MWM: Morris water maze

NCD: Normal chow diet

NFT: Neurofibrillary tangles

PHF1: Tau phosphorylated at Ser 396/404

pS214: Tau phosphorylated at Ser214

pS422: Tau phosphorylated at Ser422

ROS: Reactive oxygen species

TDP43: TAR DNA binding protein 43

Chapter 1: Introduction and Literature Review

Alzheimer's Disease and Frontotemporal Dementia

Alzheimer's disease (AD) is the most common form of dementia, representing an estimated 70% of all forms of dementia. It accounts for 60-70% of cases of dementia in people over 65 years of age, and 34% of dementia cases in people under 65 years of age[1,2]. The number of reported diagnosed cases of dementia in Australia is ~400,000, and it is expected to grow with the increase of the aging population of Australia in future years[3]. AD can be either sporadic or familial, with the sporadic variant being the most common (more than 90%)[4]. Familial AD is associated with mutations in various genes including *APP* (Amyloid Beta Precursor Protein), *ApoE* (Apolipoprotein E), and *MAPT* (microtubule-associated protein Tau)[5]. AD has an insidious onset, and along with meeting the criteria for all causes of dementia including impairment in learning and recall of recently learned information; deficits in language (in word-finding), visuospatial cognition (object agnosia, impaired face recognition, simultanagnosia, and alexia), and executive dysfunction (impaired reasoning, judgement, and problem-solving) are also observed[2]. Neuropathologically, AD is characterised by extracellular amyloid-beta protein deposition contributing to senile plaques and intracellular hyperphosphorylated protein tau deposition contributing to neurofibrillary tangles (NFTs) within neurons[1].

Frontotemporal dementia (FTD) is the second most common form of dementia, especially in people under 65 years (early-onset), accounting for 10-20% of all dementia cases[6–9]. Currently, in Australia, between 3,500 to 11,500 people are estimated to be affected by FTD[10]. FTD comprises three clinical subtypes: behavioural variant frontotemporal dementia (bvFTD), aphasic variant primary progressive aphasia (avPPA), and semantic variant primary progressive aphasia (svPPA). Clinical presentation of FTD includes persistent changes in behaviour and interpersonal functioning, which manifests as disinhibition, apathy, loss of empathy, altered

food preferences and executive deficits. In addition, some present with memory impairment and progressive aphasia[11]. Neuropathology of FTD typically involves either the neuronal cytoplasmic deposition of TAR DNA binding protein 43 (TDP43) or deposition of the protein tau mainly in the frontotemporal cortex of the brain[6] and generally, the clinical syndromes do not correlate with a specific pathology[6]. Furthermore, FTD exists on a disease spectrum with Amyotrophic lateral sclerosis (ALS) also known as motor neuron disease (MND), a debilitating neurodegenerative disease that is characterised by loss of upper and lower motor neurons in brain and spinal cord resulting in progressive muscle weakness and paralysis leading to death. FTD and ALS share overlapping pathology (neuronal inclusions containing TDP43), clinical presentations and causative genetic mutations[6].

Despite numerous clinical trials and ongoing research, there are no curative, disease-modifying or preventative treatments available for either AD or FTD, and at present, their treatments are limited to symptomatic management with acetylcholine esterase inhibitors (AchEIs) and NMDA receptor antagonists for AD, or with selective serotonin re-uptake inhibitors (SSRIs) for FTD[1,12].

Given that both AD and FTD patients exhibit tau neuropathology, both diseases belong to a group of neurodegenerative disorders called tauopathies, which is characterised by increased expression of abnormally hyperphosphorylated protein tau and its deposition and aggregation into neurofibrillary tangles (NFTs)[1].

Microtubule associated protein tau

Tau is a microtubule-associated protein (MAP), encoded by the *MAPT* gene, which is abundantly expressed primarily in neurons. Similar to other MAPs, tau interacts with tubulin

to promote its assembly into microtubules (MT) and stabilises the neuronal MT network of the central nervous system[13,14]. MTs are cylindrical structures made of tubulin that provide structure and shape to eukaryotic cells. They are a major component of the cytoskeleton and involved in mitosis, cell motility, intracellular transport of vesicles, proteins, lipids, and other organelles. In neurons they aid signal reception and transmission and act as "information carriers"[14,15]. Regulation of tau, both on and off the MTs, is dependent upon its phosphorylation state. Under pathological conditions, hyperphosphorylated tau destabilises and disassembles the MT network by disengaging excessively from the MTs. Increased cytosolic concentration of unbound tau facilitates its polymerisation and misfolding which leads to the formation of NFTs. Hyperphosphorylated tau along with NFTs sequester normal tau and other MAPs and cause axonal transport defects by becoming physical obstacles to vesicles and other cargo transported through MTs along the axons. This results in neurotoxicity which precedes neurodegeneration. Protein tau-associated mechanisms, together with other pathological events such as amyloid β-mediated toxicity, oxidative stress and inflammation are thought to initiate or contribute to the underlying pathogenesis of these neurodegenerative tauopathies[14,16,17]. In AD, tau is approximately three-to four-fold more hyperphosphorylated than in the normal adult brain[17] and tau pathology is found mostly within the entorhinal and trans-entorhinal regions of the cortex[13]. In FTD, around 40% of cases are found to have tau pathology and atrophy of the frontotemporal cortex of the brain with neuronal loss, gliosis and spongiosis[18]. Cases of FTD with tau pathology include tau-positive inclusions, ubiquitin-positive inclusions, tau-negative inclusions and those without distinctive histopathology[19].

Models depicting tau pathology in AD and FTD

Many animal models (mostly rodents) exhibiting tau pathology and a variety of phenotypes exist currently (Table 1), with variations in their promoter, tau isoform and mutation, and transgene integration site and copy number[20]. Although none of the available models are

capable of reproducing all the features of tau pathology seen in human AD and FTD brains, they have been invaluable in gaining insights into underlying disease mechanisms which in turn contribute to research geared towards discovering therapeutics[14].

In this study, the mouse model TAU58/2 which has been previously established and characterised in the Dementia Research Centre (DRC) at Macquarie University was used[21,22]. These mice express the human 0N4R tau isoform with the P301S mutation under the control of mouse neuronal Thy1.2 promoter[21,22]. P301S is a mutation in the *MAPT* gene which causes familial frontotemporal lobar degeneration (FTLD-tau). This model recapitulates features of human FTLD-tau and AD pathology where early tau pathology induces functional deficits in neurons associated with NFT pathology that is specific to tau [22]. This mouse model has reported motor deficits starting from around the age of 2-3 months old and cognitive deficits starting from 6 months of age which worsen with age. Tau phosphorylation and NFT formation were seen as early as 3 months and increased significantly with age in these TAU58/2 mice. This model also reported behavioural changes including disinhibition-like behaviour in the elevated plus maze and hyperactivity in the open field arena. The amygdala was a primary and early site of pathological tau deposition in these mice, which resembles human FTD [21].

Gene	Mutation/construct	Promoter	Pathology	Refs
MAPT	4R/2N isoform	Thy1	Hyperphosphorylated PHFs	[23]
			Axonopathy without formation of neuronal NFTs	[24]
			Axonopathy containing neurofilament- and tau-immunoreactive spheroids, especially in the spinal cord Foetal	[25]
	Foetal tau (3R/0N isoform) Prion	Prion protein promoter	NFTs in the brain at 18 months of age	[26,27]
	P301L	Prion protein promoter	Tangle pathology detectable at 2.5 months of age	[28,29]
	P301L	Thy1.2	Tangle pathology detectable at 3 months of age	[30]
	Inducible overexpression of P301L	Ca2+ –calmodulin-dependent kinase II	Tangle pathology detectable at 2.5 months of age	[31]
	Genomic tau	Endogenous	Tau-immunoreactive axonal swellings	[32]
	G272V	Prion protein promoter	Oligodendroglial fibrillary lesions	[33]
	P301S	Thy1.2	Tau pathology detectable at 5 months of age	[34]
	G272V P301L R406W	Thy1	Tau pathology detectable at 1.5 months of age. No motor impairment observed for up to 12 months after birth	[35]
	V337M	PDGF-β	Mutant tau induces neuronal degeneration, associated with the accumulation of RNA and phosphorylated tau R406W	[36]
	R406W	Ca2+ –calmodulin-dependent kinase II	Hyperphosphorylated tau inclusions appear	[37]

			in the forebrain at 18 months of age. No motor abnormalities for up to 23 months after birth	
	G272V P301S	Thy1.2	Hyperphosphorylated tau, tangles and PHFs. No motor impairment for up to 18 months after birth	[38]
	P301S	Prion protein promoter	This model recapitulates tauopathy, including early indications of degeneration, such as synapse loss and microglia activation	[39]
Apolipoprotein E (ApoE)	ApoE4	Multiple	Phosphorylated tau expression in the neocortex, the hippocampus and the amygdala	[40]
Cdk5r1	p25	Neuron-specific enolase	Phosphorylated tau expression in the cortex, the amygdala and the thalamus	[41]
Amyloid beta precursor protein (APP), Presenilins1 (PSE1), MAPT	APP695(Swe), PS1(M146V), and Tau(P301L)	Thy-1 promoter	Phosphorylated tau and tangle pathology in the cortex and hippocampus, accumulation of intraneuronal Aβ in the hippocampus and amygdala.	[42]
Amyloid beta precursor protein (APP), Familial Alzheimer's disease (FAD)-associated genes, Presenilins1 (PSE1), MAPT	Swedish mutations(K670N/M671L), two FAD-associated mutations (I716V/V717I), PS1(M146V and L286V)	Thy-1 promoter	Aβ plaques in the cortex and hippocampus, tau pathology initially in locus coeruleus, trans-entorhinal cortex, then hippocampus and higher cortical regions	[42]

Table 1: Commonly used transgenic mouse models of tau pathology. Adapted from Ballatore et al and Lee et al[14,42]

Inflammation and neurodegeneration

Neuroinflammation is considered a pathological hallmark feature of neurodegenerative diseases such as AD and FTD[18]. It involves resident innate immune glial cells of the CNS, mainly microglia and astrocytes causing gliosis[18,43]. Dysregulation, misfolding or accumulation of tau, amyloid beta, or TDP43 is thought to induce a cytotoxic response from the glial cells which causes the release of pro-inflammatory factors such as C-reactive protein, cytokines and chemokines which cause mitochondrial dysfunction, oxidative stress damage and synaptic impairment. Prolonged glial activation therefore causes chronic inflammation which ultimately leads to neurodegeneration[18,43].

Oxidative stress is defined as the disturbance of balance between pro-oxidant and antioxidant levels, and it is caused by a group of naturally occurring chemically reactive pro-oxidant molecules called Reactive oxygen species (ROS) generated from mitochondria, NADPH oxidase, and xanthine oxidase. ROS are maintained at comparatively low levels by endogenous antioxidants[44], however, this balance can be disrupted by mitochondrial dysfunction and/or neuroinflammation which are triggered by the accumulation of misfolded proteins as seen in AD and FTD[44].

The NLRP3 inflammasome, in particular, has become a key focus of research into inflammation in neurodegenerative diseases, specifically in AD. This inflammasome is a multiprotein complex which is a critical component of the innate immune system that mediates inflammatory responses and programmed cell death[45]. In AD, the NLRP3 inflammasome has been shown to be activated in response to Aβ accumulation and tau pathology, and causes the generation of pro-inflammatory factors (caspase-1-mediated interleukin (IL)-1β and IL-18) in microglia, which causes mitochondrial dysfunction, oxidative stress damage, and ultimately neurodegeneration[45]. Currently, the NLRP3 inflammasome is believed to be an important therapeutic molecular target for AD as it may be regulating neuroinflammation[45,46].

Dietary interventions in neurodegenerative diseases

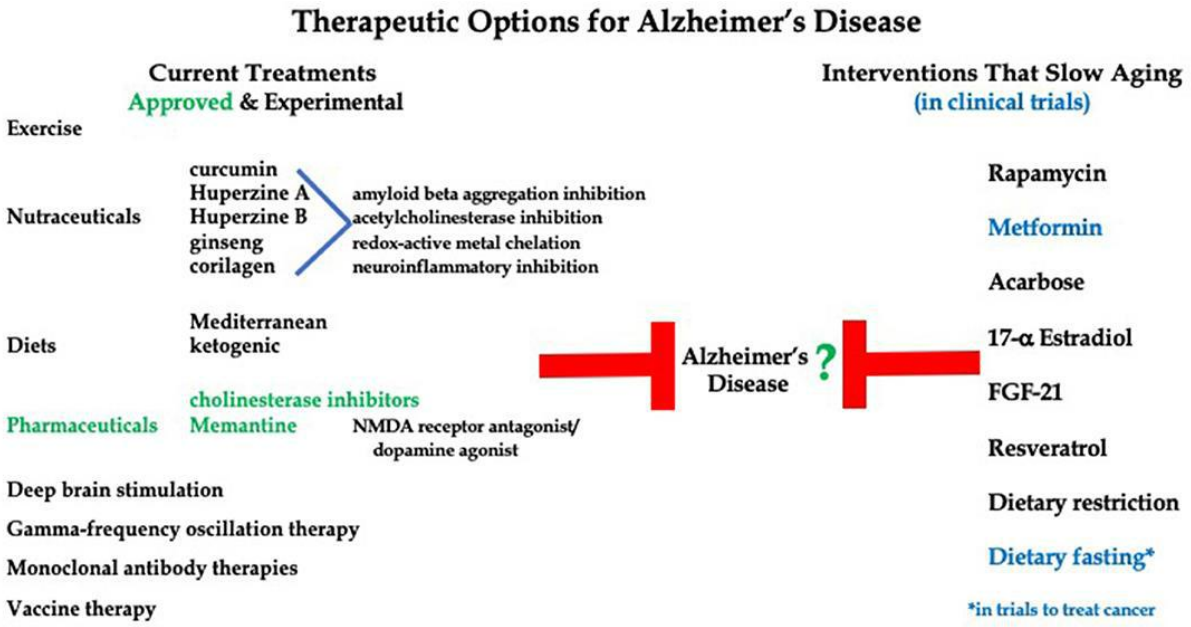

Figure 1: Current therapies to treat Alzheimer's Disease symptoms and pathologies versus potential therapies for AD. Adapted from Thelen et al[47]

Since there are no curative or preventative treatments available and both AD and FTD are highly progressive diseases, the burden and cost of care increases with a patient's lifetime. Therefore, patients and caregivers have turned to adapting alternative approaches (see Figure 1 for examples) such as exercise and lifestyle modification, nutraceuticals, ketogenic/high-fat diets, and the Mediterranean diet[47,48]. Coconut oil (CO), being a naturally occurring ketogenic Ingredient that can be[49–51] easily and cost-effectively integrated into one's diet, has been strongly promoted by the media, even though quality evidence from randomised, placebo-controlled clinical trials is lacking.

Successful outcomes such as a reduction of symptoms and slowing down of disease progression have been reported using ketogenic or high fat diets in many neurological diseases such as epilepsy and stroke rehabilitation[52]. These studies suggest that by providing ketone bodies as an alternative fuel to neurons, the energy imbalance which occurs due to hypometabolism, and

mitochondrial deficits present in the neurodegenerative brain can be restored. Neuroketotherapeutics, a class of bioenergetic therapies that use the induction of ketosis, have also shown potential in the treatment of AD[52,53] and in age-related neurodegenerative disorders including AD, FTD, Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (ALS) according to a review by Cunnane et al[54].

The debate on the ability of these ketogenic diets to improve outcomes in age-related neurodegenerative diseases is still very much ongoing. Reported adverse effects of ketogenic diets include muscle cramps, bad breath, changes in bowel habits, keto-flu, loss of energy and an inability to sustain energy levels due to their low levels of carbohydrates, an important energy source[55]. There is also evidence of micronutrient deficiency[56] and cardiovascular risk[57]. Therefore, utilizing these diets in the hopes of improving cognition in neurodegeneration should be carefully considered against how they affect general overall health.

A study conducted in two mouse models; APP/PS1 (a model of amyloid deposition) and Tg4510 (a model of tau deposition) mice of AD showed that a ketogenic diet improved motor performance (rotarod), but not cognition (Y-maze, fear conditioning, and radial arm water maze)[58]. However, a double-blind placebo study and a pilot intervention study which were conducted on two groups of memory impaired patients with probable AD (with and with-out APOE-ε4 allele) have indicated cognitive improvement with a ketogenic diet[59,60]. Data indicate that ketogenic diets also alleviative effects on age-related neurodegenerative diseases by enhancing mitochondrial respiration, promoting neuronal long-term potentiation, decreasing oxidative stress, and reducing inflammation[46,52].

Research has been conducted on the benefits of adhering to the Mediterranean diet, a diet inspired by the eating habits of people of Mediterranean countries which is "characterised by a high consumption of fruit, vegetables, legumes, and complex carbohydrates, with a moderate

consumption of fish, and the consumption of olive oil as the main source of fats and a low-to-moderate amount of red wine during meals."[48] in AD as it has been observed to be associated with lower risk for mild cognitive impairment (MCI), conversion of MCI to AD, and lower mortality in AD [61–64]. Furthermore, there is evidence that adherence to a Modified Mediterranean diet known as the MIND diet (the Mediterranean diet and the Dietary Approaches to Stop Hypertension: DASH diet) lowers dementia risk. The MIND diet encourages the consumption of green-leafy vegetables, all other vegetables, berries, nuts, olive oil, whole grains, fish, beans, poultry, and wine (in moderation) and avoidance of butter and margarine, cheese, red meat, pastries and sweets, and fried food[65,66].

In addition, in patients suffering from neurodegeneration, especially FTD, changes in their dietary behaviour is observed, where marked hyperphagia, rigid eating behaviour (eating the same foods repeatedly) and changes in food preference are reported[67]. A series of recent experiments performed on a group of bvFTD and AD patients who met current clinical diagnostic criteria for probable bvFTD, semantic dementia, or AD, revealed that differing neural networks which control eating behaviour and sucrose preference are responsible for hyperphagia and increase of sucrose consumption in patients with bvFTD and semantic dementia[68]. They found that these eating abnormalities are multifactorial and are partly related to hypothalamic degeneration, with potential disintegration of the network connections between the hypothalamus and orbitofrontal cortex/reward pathways[69]. Another study also revealed that bvFTD patients showed increased preference for high-fat meals compared to AD patients and control participants[70]. Therefore, according to these studies, a tendency towards high caloric, high fat diets in patients with neurodegenerative diseases is apparent, and it would be worthwhile to see if adhering to a similar diet would potentially help in improving disease progression or provide an acceptable treatment target.

Basis of research on coconut oil-derived fat diets for AD and FTD

Neurons depend on adipose triphosphate (ATP) generated from glucose metabolism (which occurs in mitochondria) for their cellular energy requirements. In the neurodegenerative landscape of AD and FTD, mitochondria are less able to absorb glucose, and therefore cerebral glucose metabolism is reduced. In this instance, ketones generated in the liver from the breakdown of medium-chain triglycerides (MCT) which contain medium-chain fatty acids, can act as a substitute for glucose, providing neuronal mitochondria with an energy source[51,71]. Coconut oil (CO) contains the medium-chain fatty acids caprylic acid, capric acid, and lauric acid[51,71]. It is also a natural antioxidant-rich compound[72] with anti-inflammatory and lipid-lowering qualities[73], which add to the suitability of CO as a likely candidate for reduction of symptoms of AD, FTD and related tauopathies.

Although there is much interest in the role of ketogenic diets in neurodegeneration and there are many studies supporting their benefits in AD, studies done specifically on CO or CO derivatives are lacking. For example, Reger et al. and Taylor et al. performed studies in AD patients on MCT-supplemented diets, and showed significant improvements in Alzheimer's Disease Assessment Scale-cognitive subscale scores (ADAS-Cog: assesses the level of cognitive dysfunction in Alzheimer's disease)[59,60]. However, a randomized double-blind placebo-controlled pilot intervention study was conducted on a group of AD patients who had either not previously received treatment or had been stabilized on cholinesterase inhibitors or memantine for at least 3 months prior to the trial, and who had a mini-mental state examination (MMSE) score of 10-24. Patients who consumed cold-pressed coconut oil did not show any significant improvement in cognition or behaviour[74].

A handful of laboratory studies have also demonstrated neuroprotective effects of CO and its derivatives on amyloid beta (Aβ) pathology. Maltolyl p-coumarate, an ester of p-coumaric acid (found in CO) and maltol, has been shown to decrease cognitive deficits in Aβ42-infused rats[75];

and in rats injected with Aβ42, where the administration of p-coumarate reduced levels of apoptosis [75]. In another study by Yan et. Al, the CO-derived phenolic compound ferulic acid lowered cortical Aβ levels in an AD transgenic mouse model[76]. In a study performed on rats receiving Aβ and a high-fat diet, a diet fortified by virgin coconut oil seemed to normalise NLRP3 inflammasome and showed potential neuroprotective effects[73]. A study utilising an edible dairy formula fortified with CO given to rats with aluminium chloride-induced Alzheimer's disease revealed that it ameliorated cognitive impairment, reduced oxidative stress markers and restored the architecture of the brain[77].

Chapter 2: Aims and Hypothesis

Although the above-mentioned laboratory animal studies have shown benefits of coconut oil (CO) for AD with Aβ pathology[73,75–77], research on CO and FTD and CO on overall tau pathology are non-existent. Therefore, further research on the effects of CO on tau-mediated neurodegenerative diseases is warranted to potentially identify CO as a beneficial alternative therapeutic agent for these dementias.

By conducting this research, the current study aimed to provide evidence that a diet based on CO may alter tau pathology and improve memory impairment in the TAU58/2 mouse model of AD/FTD. The main objective of this study is to investigate whether specialised high fat diets consisting of either coconut-derived fat or lard result in differences in hippocampal function, spatial memory and neuropathology in TAU58/2 transgenic and wild-type mice compared to matched mice fed with a control standard chow diet.

Therefore, the specific aims of this study are to:

1) Analyse cognitive domains of learning and memory using the Morris Water Maze (MWM) by assessing swim path strategies of TAU58/2 mice and wild-type controls who are fed specialised diets consisting of coconut-derived or lard-derived fat compared to control mice fed a standard chow diet.
2) Determine the extent of tau neuropathology and neuroinflammation within the post-mortem brain of TAU58/2 mice and wild-type controls fed specialised diets consisting of coconut-derived or lard-derived fat compared to control mice on a standard chow diet utilising histological techniques.

Therefore, the hypothesis tested is:

P301S mutant tau transgenic (Tau58/2) mice, when fed with a diet of coconut-derived fat, perform better in Morris Water Maze (MWM) test. Thus, they show better hippocampal

function related to learning and retained memory, and have a reduction in the pathological protein tau deposition and neurofibrillary tangles (NFTs), compared to a control who are fed a normal diet, or a high-fat diet based on lard.

Chapter 3: Methods and Materials

Mouse model

An FTD mouse model established and characterised previously in the Dementia Research Centre (DRC) was used for this study. TAU58/2 transgenic mice over-express the human 0N4R tau isoform with the P301S mutation[22,78].

The mice were put on high fat diets consisting of coconut-derived (D12331) or lard-derived fat (D12492), or standard chow diet from weaning (3 weeks of age) until the end of the experiment (see Table 2 for fat content and sources). Mice had *ad libitum* access to food and water and were maintained on a 12-hour light/dark cycle. For each diet, a cohort of transgenic TAU58/2 (n=3-8) and wildtype littermates (n=7-9) were used. Sample sizes were decided based on similar previous experiments[1,2]. None of the mice were excluded during the experiment and all mice which were healthy at weaning (prior to going on the diets) were included in the study up to the required n number. With regards to randomisation, mice were weaned into full cages and all mice in the same cage went on the same diet; e.g. 1st cage: standard chow diet , 2nd cage: coconut-derived fat diet, 3rd cage: lard-derived fat diet.

All animal experiments were approved by the Animal Ethics Committees of Macquarie University and the University of New South Wales. All procedures complied with the statement on animal experimentation issued by the National Health and Medical Research Council of Australia. This experiment did not subject animals to any stress, suffering or distress. No adverse events were reported due to the experimental procedure. The mice were monitored once weekly according to ethical process (additionally cages were checked every day by facility staff). Refer to appendix for the monitoring sheets that were used in the study.

Diet	Fat content (gm)%	Fat sources
D12331	35.8	Soybean oil, Hydrogenated coconut oil
D12492	34.9	Soybean oil, Lard
Meat free rat and mouse diet	4.6	Mixed vegetable oils, Canola oil (fixed formula)

Table 2: Diet information. Open formula purified diets for lab animals, Research diets, Inc. Refer to appendix for further information on diet.

Cognitive testing: Morris water maze

Morris Water Maze (MWM) data obtained previously in the DRC by Dr Annika van Hummel was used for this thesis. For MWM testing, Forty-three male, 3.5-month-old TAU58/2 mice (n=19) and wildtype littermates (n=29) were placed in a circular tank of 1500mm in diameter filled with water made opaque with a white pigment. Visual cues were placed around the diameter of the pool, and mice were allowed to swim freely for 60 seconds to navigate to a submerged escape platform. Mice were allowed to familiarise themselves with maze and the task for 5 days (learning phase), and the time taken to reach the platform was recorded. Each mouse performed 4 trials per day, starting from randomised start positions in quadrant 1, and the average time per day was used for analysis of the learning curve. Each trial was recorded using a camera on the ceiling using IC Capture software (The Imaging Source, Taiwan). Following the learning phase, a probe trial was performed the next day, where the platform was removed from the pool and the mice were allowed to swim freely for 30s. The preference of the mice to reach the platform quadrant (former goal location) indicated successful spatial learning and reference memory. The data from the probe trial and learning curve were analysed using Graphpad Prism Software using one-way-ANOVA and two-way-ANOVA tests respectively.

The recorded swim path data of these mice were analysed using Anymaze video tracking software (*Stoelting, IL USA)* and manually categorised according to the strategies outlined in

Garthe et al, as seen in Figure 2 [79]. The patterns discerned by *Garthe et al* were assigned a score and each of the swim paths of the mice were scored accordingly. Swim patterns assigned as thigmotaxis, random search, and scanning were considered non-hippocampal and given the scores 1, 2, and 3, whereas swim patterns that resembled chaining, directed search, focal search, and direct swimming were considered hippocampal and were given the scores 4, 5, 6, and 7, respectively. The assessor was blinded to genotype and diet information while examining the swim paths using Anymaze software and scoring each swim path. Every swim path was scored twice by two independent researchers before a final score was discerned.

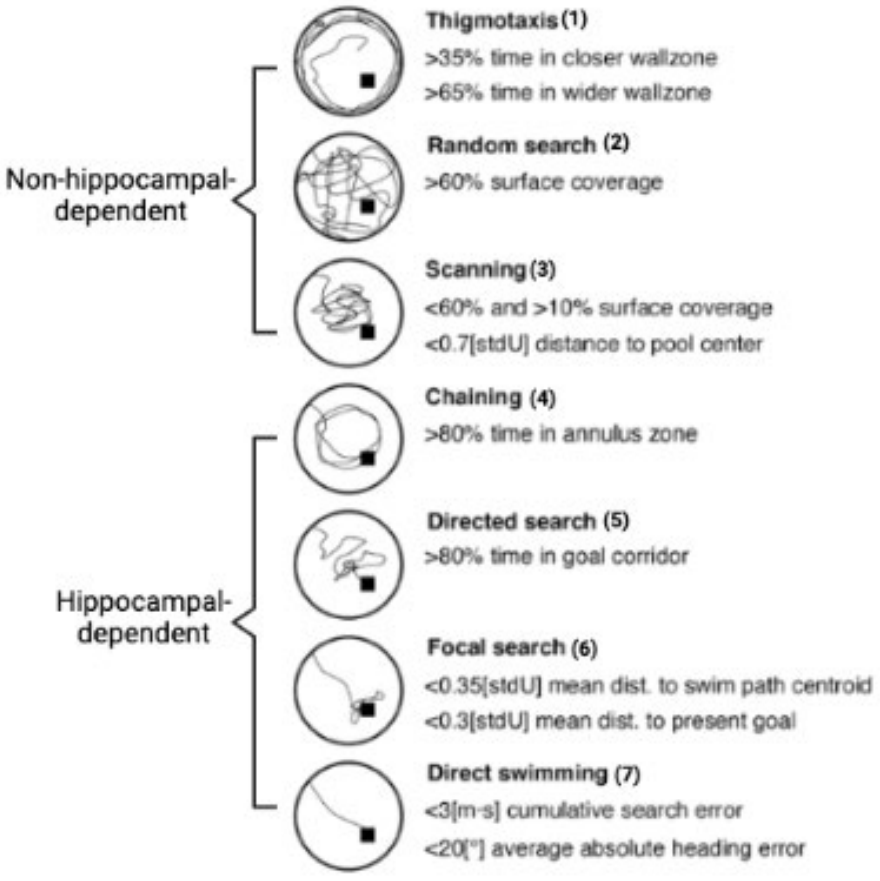

Figure 2: Hippocampal-dependent vs non-hippocampal-dependent swim paths. Adapted from Garthe et al[79]*. Thigmotaxis, Random search, Scanning (Scores 1, 2, and 3) were considered non hippocampal-dependent, whereas Chaining, Directed search, Focal search, and Direct swimming (Scores 4, 5, 6, and 7) were considered hippocampal-dependent swim paths.*

Immunofluorescent staining and microscopy

At 5 months of age, mice were transcardially perfused and brain tissue collected by Dr Annika van Hummel. Formalin-fixed paraffin-embedded (FFPE) post-mortem brain tissue from each mouse was sectioned at 4µm using an HM355S automatic waterfall microtome (Thermo Fisher Scientific) and mounted on slides (sagittal sections). Subsequent immunofluorescent staining was done according to previously established protocols[21,22,80]. Briefly, antigen retrieval using citric buffer (pH 7.4) was performed prior to blocking the sections in Goat-blocking-buffer (GBB, 3% heat-inactivated goat serum, 2% Bovine Serum Albumin in Phosphate-buffered saline) and overnight incubation at 4 degrees in primary antibody (details in Table 3). All antibodies were sourced from Thermo Fisher Scientific. The next day, slides were incubated in Alexa fluor conjugated secondary antibodies (1:250 dilution) for ∼1-2 hrs in the dark. Then they were cover-slipped and further kept in the dark, ready to be scanned.

Antibodies against target markers tau, neurofibrillary tangles, astrocytes, and glia (neuropathology, inflammation) were used for immunofluorescent staining intensity analysis.

Antibody	**Target**	**Dilution**	**Experiment**
Human Tau (Tau13)	Tau	1:500	IF, FFPE
Tau phosphorylated (PHF1) Ser 396/404	Tau	1:250	IF, FFPE
IBA1	Microglia	1:500	IF, FFPE
GFAP	Astrocytes	1:200	IF, FFPE
NeuN	Neurons	1:400	IF, FFPE
NF200	NFT's	1:500	IF, FFPE

Table 3: Antibodies used in the immunofluorescent staining.

Next, the immunofluorescent stained mouse brain tissue sections were scanned via AxioScan Z1 slide-scanner (Carl Zeiss PTY LTD) Quantification of staining (i.e., tau expression, extent of NFTs, extent of glial changes) within neurons was performed using Zen 2 blue software (Zeiss) according to previously published protocols[22,80,81]. The hippocampus was traced using the Zen Blue graphics feature and mean intensity for each antibody within the marked areas

was obtained (channel 488-green, channel 555-red, DAPI-blue) (Figure 3). The intensity data was then graphed and analysed using Graphpad Prism software version 9.0 and one-way ANOVA tests with multiple comparisons and Tukey test for corrections.

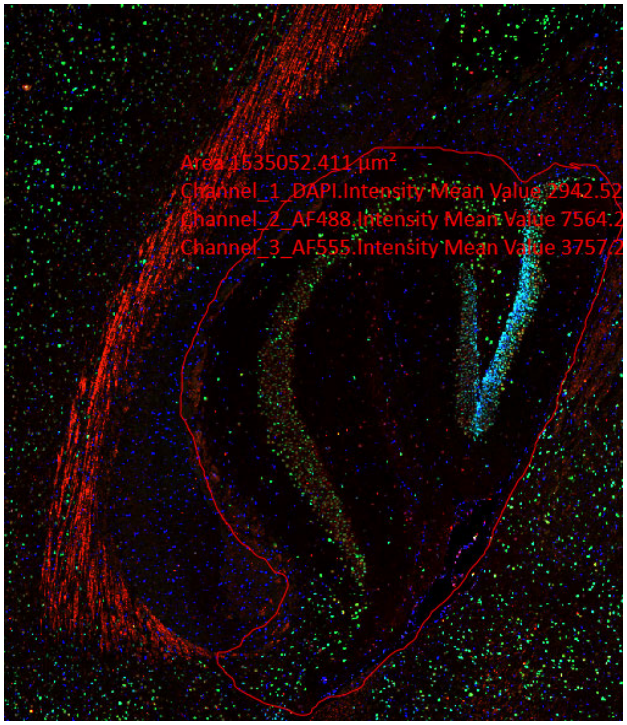

Figure 3: The hippocampus was traced using the Zen Blue graphics feature and mean intensity for each antibody within the marked areas was obtained (channel 488-green, channel 555-red, DAPI-blue)

In addition, further tissue was sectioned at 8µm thickness and stained using Gallyas silver staining/impregnation methods, as described in *Ittner et al, 2008*[78]. Gallyas silver stain is used to label the expression of NFTs. The total number of NFTs, identified by black colour, and neuronal size and shape, were counted throughout the hippocampus at and the average stained cell counts/μm^2 for each diet cohort were compared. Zeiss Zen 2 (blue edition) software was used to visualise the scanned sections and perform manual counts.

Statistics:

MWM swim path analysis scores were counted and graphed using Microsoft Excel and its formulae =BINOM.DIST, =COUNTIF, =AVERAGE, =SUM (Figure 4 A, B, C, D, E, F).The data from the learning curve were analysed by Graphpad Prism Software version 9.0 using two-way-ANOVA test with multiple comparisons and Tukey test for corrections (Figure 4 H). The

data from the probe trial were also analysed by Graphpad Prism Software using one-way-ANOVA test using multiple comparisons and Tukey test for corrections(Figure 5 A, B, C). The intensity data of the stained hippocampal areas were analysed and graphed using Graphpad Prism software and one-way ANOVA tests with multiple comparisons and Tukey test were used for corrections. P vales below 0.05 were considered significant. All values are presented as mean ± standard error of the mean. (Figures 6-14).

Chapter 4: Results

Cognitive testing revealed higher hippocampal-dependent behaviour in mice fed a coconut-derived fat diet

Morris Water Maze (MWM) testing was conducted to investigate spatial learning and memory consolidation[82–84] in TAU58/2 mice and wild-type littermates fed different diets.

By the 5th day of the learning phase, the difference in the number of hippocampal-dependent swim patterns used by the wild-type mice fed with normal chow (NCD) and transgenic mice fed with NCD was significant, with wild-type mice showing a higher amount of hippocampal-dependent swim patterns (p=0.01*) (Figure 4 A, B). Conversely, transgenic mice fed the coconut fat diet (CFD) demonstrated a significantly higher amount of hippocampal-dependent swim patterns compared to wild-type mice fed with CFD (p<0.0001****) (Figure 4 C, D). Similar to mice on the NCD, transgenic mice fed with lard diet showed significantly lower hippocampal-dependent swim patterns compared to wild-type mice on the same diet (p=0.004**) (Figure 4 E, F).

hippocampal-dependent swim patterns	p-value
NCD wild-type > NCD transgenic	p=0.01*
CFD wild-type < CFD transgenic	p<0.0001****
LD wild-type > LD transgenic	p=0.004**

When comparing the wild-type mice of each diet cohort, the difference between the wild-type mice of NCD and CFD was significant, wild-type mice of NCD exhibiting higher amount of hippocampal-dependent swim patterns (p=0.0302) (Figure 4 A, C). Wild-type mice fed with LD showed the highest amount of hippocampal-dependent swim patterns (NCD vs LD non-significant, p=0.0645, CFD vs LD p= 0.0005***) by the 5th day of trials (Figure 4 E).

hippocampal-dependent swim patterns	p-value
NCD wild-type > CFD wild-type	p=0.0302
NCD wild-type vs LD wild-type	p=0.0645 (non-significant)
CFD wild-type < LD wild-type	p= 0.0005***

Most importantly, by the 5th day of trials, when comparing the transgenic mice of each diet cohort, transgenic mice in the CFD group performed significantly better than both NCD and LD in the amount of hippocampal-dependent swim patterns ($p<0.0001$****) (Figure 4 D). There was no difference between transgenic mice in the NCD and LD groups over the 5 days of learning curve ($p=0.1$).

hippocampal-dependent swim patterns	p-value
CFD transgenic > NCD transgenic	p<0.0001****
CFD transgenic > LD transgenic	p<0.0001****
NCD wild-type < LD transgenic	p=0.1 (non-significant)

Overall, transgenic mice of the CFD group shows the highest average scores. The difference between them (CFD Tau58) and transgenic mice fed with NCD was significant (CFD Tau 58> NCD Tau58= $p<0.01$) (Figure 4 G). Highest scores in the experiment were attributed to hippocampal swim patterns. Therefore, higher the average score of a group, better the performance of the group with regards to hippocampal swim patterns.

Analysis of the average time (of 4 swims) each mouse took to find the platform each day for 5 days (Figure 4 H) also known as the learning curve, revealed that by Day 5, the transgenic TAU58/2 coconut-derived fat diet cohort took the least amount of time to find the platform, indicating a higher learning capability compared to mice in other diet groups. It revealed that

there was no significant difference between the average times it took for the wild-type and transgenic mice of NCD to find the platform (p=0.06). Similarly, mice fed with LD did not have a significant difference between the wild-type and transgenic mice in the average time it took for them to find the platform. However, between the wild-type and transgenic mice of CFD, there was a significant difference in the amount of time it took for them to find the platform (p=0.043*), where the transgenic mice of CFD found it faster than their wild-type mice indicating a clear improvement in learning. The most interesting finding was that transgenic mice of CFD found the platform is in a significantly less time than transgenic mice of CFD (p=0.005**). There was no significant difference between the amounts of time taken to find the platform by transgenic mice fed with NCD and transgenic mice fed with LD.

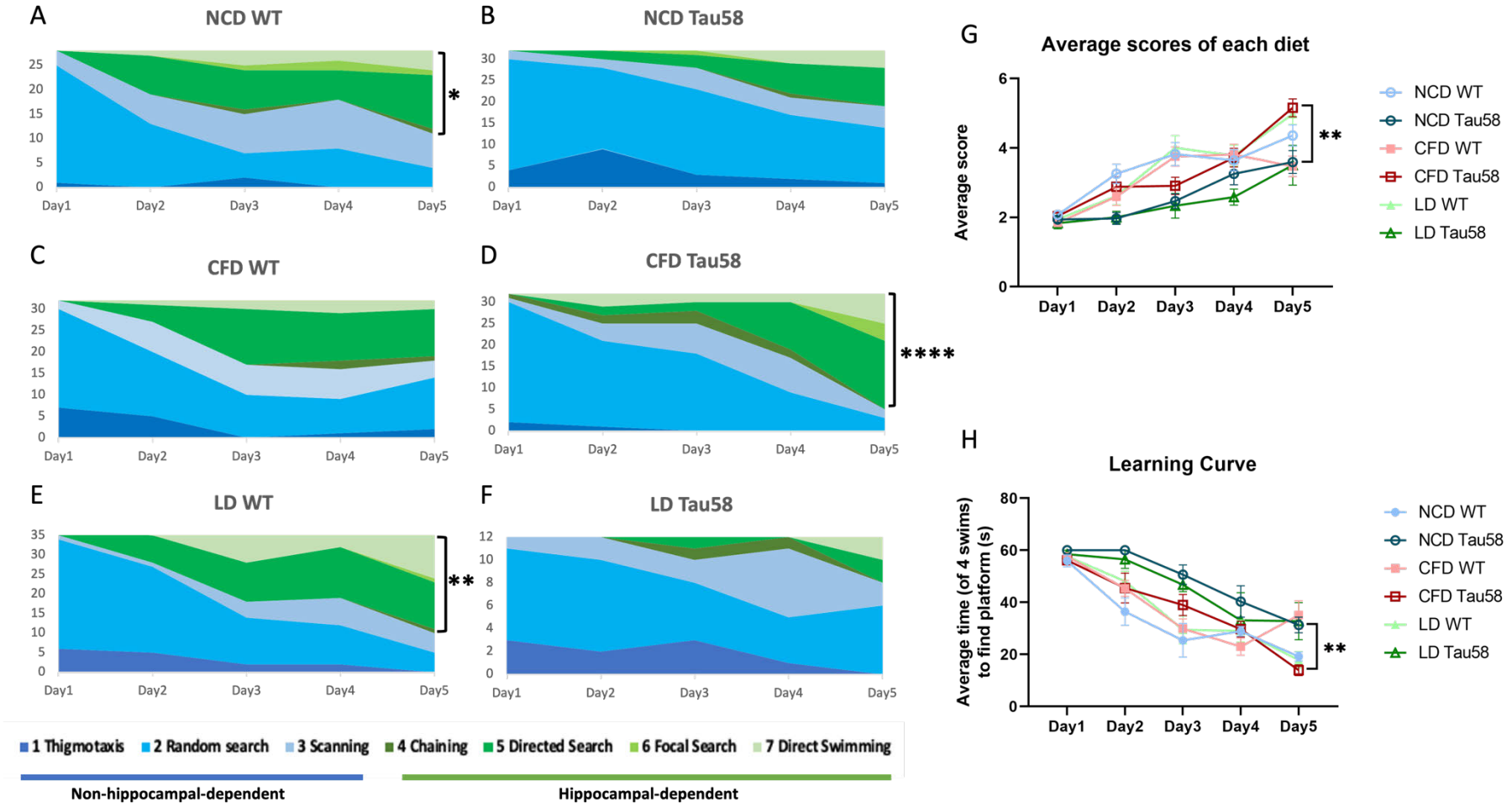

*Figure 4: MVM swim path analysis of transgenic (Tau58) and wildtype (WT) Tau58/2 mice fed with coconut-derived fat diet (CFD), lard diet (LD), or normal chow diet (NCD). Graphs A-F show the number of swims per day assigned to each of the 7 swim strategies. (A) Wild-type mice fed with normal chow diet. (B) Transgenic mice fed with normal chow diet. (C) Wild-type mice fed with coconut-derived fat diet. (D) Transgenic mice fed with coconut-derived fat diet. (E) Wild-type mice fed with lard diet (F) Transgenic mice fed with lard diet, NCD WT >NCD Tau58 (A>B): p=0.01, CFD WT <CFD Tau58 (C<D): p<0.0001, LD WT>LD Tau58 (E>F): p=0.004, (G) Analysis of the average scores of each diet. (H) Analysis of the average time (of 4 swims) each mouse took to find the platform each day for 5 days (Learning curve) (NCD WT n=7, NCD Tau58 n=8, CFD WT n=8, CFD Tau58 n=8, LD WT n=9, LD Tau58 n=3, data in G and H are shown as mean+SEM, individual data points are represented by the shapes. *=p<0.05,**=p<0.01, ****=p<0.0001*

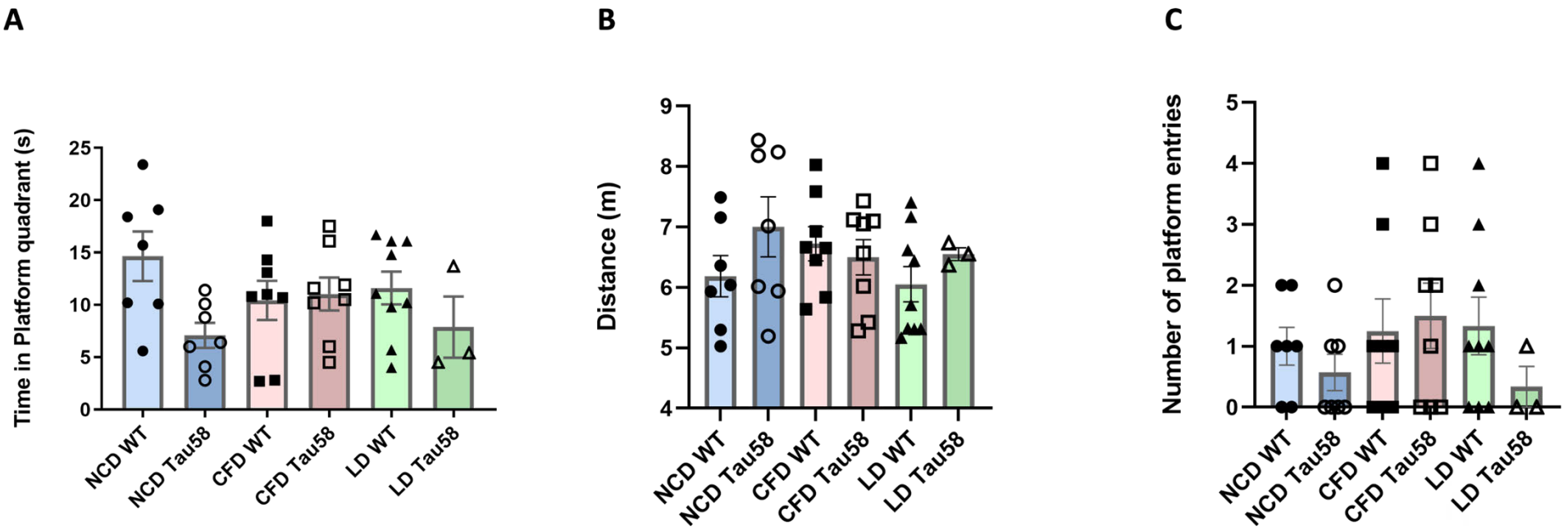

Figure 5: MWM Probe trial data: (A)time each mouse spent in the platform quadrant, (B) the distance travelled during the probe trial, and (C) the number entries to the platform quadrant in Tau58/2 mice (Tau58) and wild-type littermates (WT) fed coconut-derived fat diet (CFD), lard diet (LD), or normal chow diet (NCD) (NCD WT n=7, NCD Tau58 n=8, CFD WT n=8, CFD Tau58 n=8, LD WT n=9, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes).

The probe trial was conducted following the learning phase where the platform was removed from the MWM tank, and the mice were allowed to swim freely for 30s and data on the time each mouse spent in the platform quadrant, the distance travelled, and the number entries to the platform quadrant were obtained (Figure 5 A, B, C). Analysed results from the probe trial data, which would have been an indication of spatial bias and reference memory[85], did not show any significant difference across the three diet groups.

The difference in time spent in the platform quadrant between the transgenic mice fed with CFD and the wild type mice of the same cohort was not significant (p= 0.999), and so were the differences in time spent in the platform quadrant between the transgenic mice and wildtype mice of LD (p= 0.856) and NCD (p= 0.064), even though NCD wildtype mice tended to spend more time in the platform quadrant than NCD transgenic mice. The amount of time spent in the platform quadrant was not significantly different when comparing wild type mice on NCD and CFD (p= 0.558), NCD and LD (p= 0.817), or CFD and LD (p= 0.996). There were also no differences between transgenic mice regardless of diet, NCD and CFD (p= 0.625), NCD and LD (p= 0.999), CFD and LD (p= 0.927). Similarly there were no differences observed in the time spent in the platform quadrant between wildtype mice and TAU58/2 mice within each diet (CFD p>0.997, LD p=0.964, NCD p=0.570).

In addition, there were no significant differences in the distance travelled in the probe trial between wildtype mice or transgenic mice in each of the diet cohorts (NCD p=0.569, CFD p=0.976, LD p=0.963), or between diets (NCD vs CFD p=0.895, NCD vs LD p=0.979, CFD vs LD p>0.999). Similarly there were no significant differences in the number of entries to the platform quadrant between wildtype mice or transgenic mice in each of the diet cohorts (NCD p=0.986, CFD p=0.998, LD p=0.832), or between diets (NCD vs CFD p=0.703, NCD vs LD p=0.999, CFD vs LD p=0.736).

Neuronal transgene expression in TAU58/2 mice

Tau is abundantly expressed physiologically in neurons. Pathologically hyperphosphorylated tau is observed in AD and FTD[14]. In the current study P301S mutant human tau transgene expression was observed in the hippocampus of TAU58/2 mice using immunofluorescent staining with antibodies targeted to human tau (Tau13).

Immunofluorescent staining with Tau13 antibody within the hippocampal area of TAU58/2 mice of each diet cohort was performed (Figure 6) and analysis comparing mean intensity expression between groups showed that the intensity of human tau expression in transgenic mice of each diet cohort is significantly higher compared to expression in wild-type mice on the same diet (Figure 6 G), $p<0.0001$****). However, there were no significant differences in mean intensity expression between transgenic mice when comparing the three diets (NCD vs CFD $p=0.885$, NCD vs LD $p=0.963$, CFD vs LD $p>0.999$).

Immunofluorescent staining of antibodies targeted to the expression of postmitotic neuronal cells (NeuN) (Figure 7) within the hippocampal area did not show any significant differences in staining intensity among the three diet groups (NCD $p=0.998$, CFD $p=0.235$, LD $p=0.811$) or between the transgenic and wild-type mice within the same diet group (NCD vs CFD $p=0.488$, NCD vs LD $p=0.509$, CFD vs LD $p=0.999$).

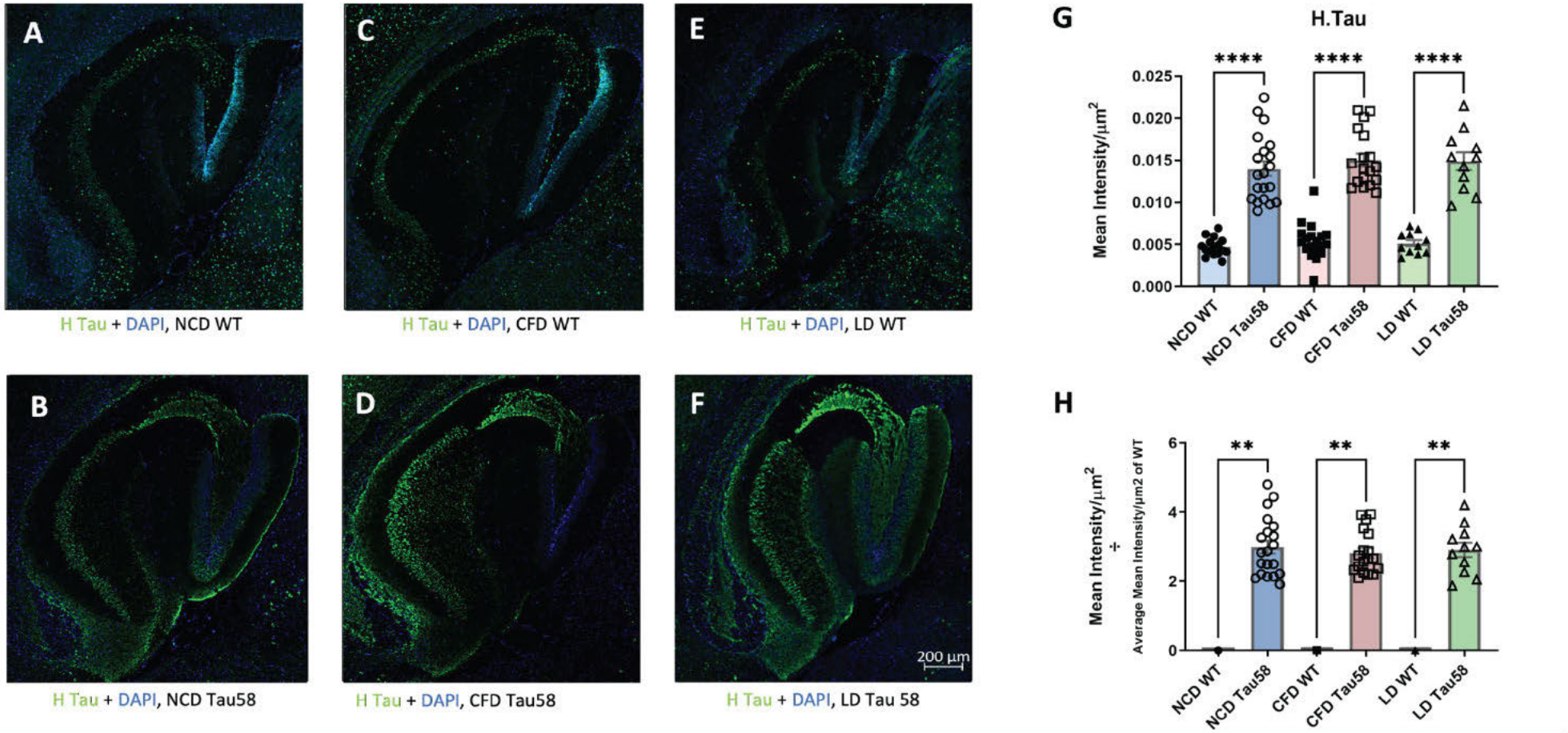

*Figure 6: Representative images of human tau in the hippocampal area of TAU58/2 mice. (A) Immunofluorescent staining of antibodies targeted to human tau (Tau13, green) in normal chow diet (NCD) wildtype (WT) mice, (B) Immunofluorescent staining of antibodies targeted to human tau (Tau13) in NCD TAU58/2 mice (Tau58), (C) Immunofluorescent staining human tau (Tau13) in coconut-derived fat diet (CFD) WT, (D) Immunofluorescent staining of human tau (Tau13) in CFD Tau58, € Immunofluorescent staining of human tau (Tau13) in lard diet (LD) WT, and (F) Immunofluorescent staining of human tau (Tau13) in LD Tau58. (G)Mean intensity of human tau in the hippocampus normalised to area, (H)Mean intensity of human tau in the hippocampus normalised to area relative to average mean intensity of WT, **=p<0.01****=p<0.0001 (NCD WT n=7, NCD Tau58 n=8, CFD WT n=8, CFD Tau58 n=8, LD WT n=9, LD Tau58 n=3, data in G are shown as mean+SEM, individual data points are represented by the shapes, DAPI-stained cell nuclei are in blue).*

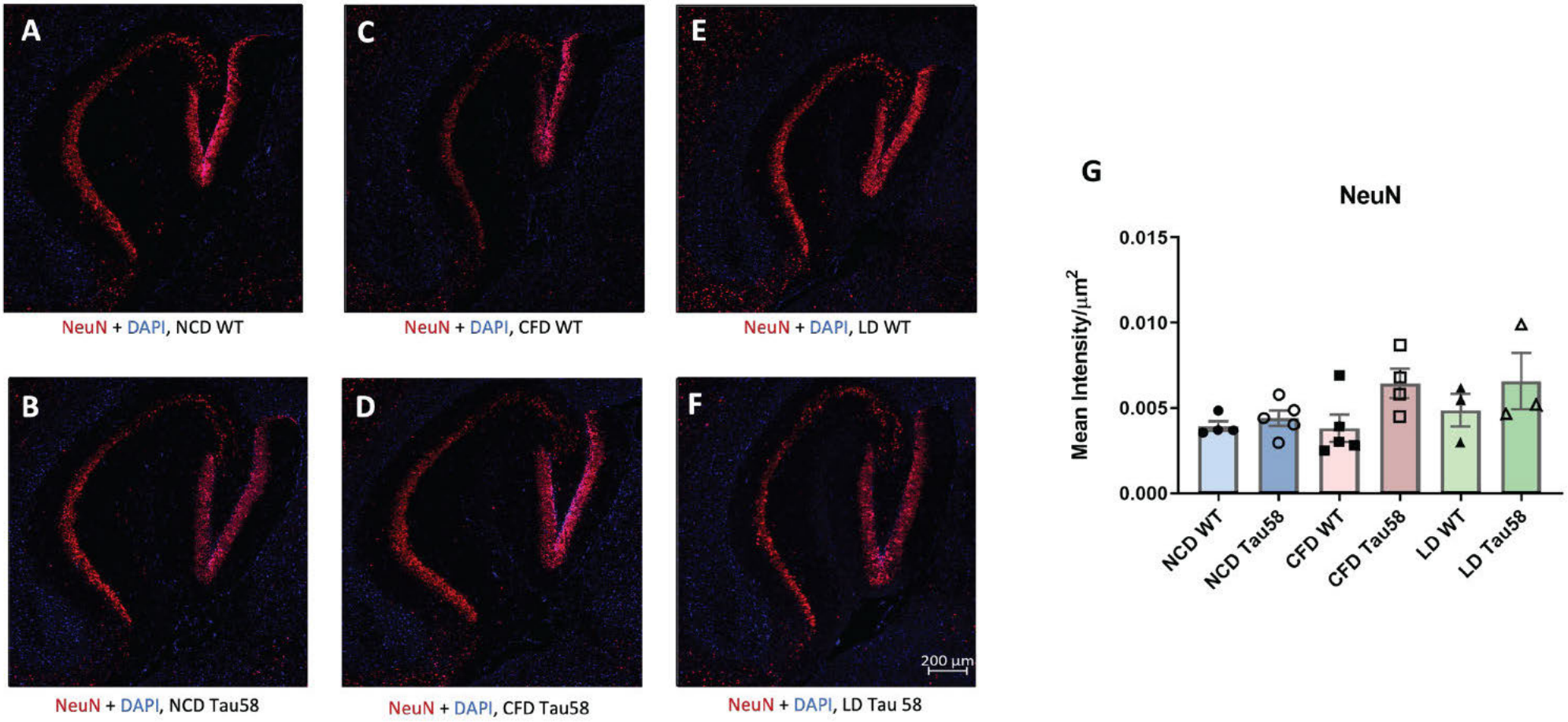

Figure 7: Representative images of neurons (NeuN) in the hippocampal area. (A) Immunofluorescent staining of NeuN neuronal marker (red) in normal chow diet (NCD) wildtype (WT) mice, (B)Immunofluorescent staining of NeuN in NCD TAU58/2 (Tau58), (C)Immunofluorescent staining of NF200 in coconut fat derived (CFD) WT, (D)Immunofluorescent staining of NeuN in CFD Tau58, (E)Immunofluorescent staining of NF200 in lard diet (LD) WT, and (F)Immunofluorescent staining of NF200 in LD Tau58. (G) Mean intensity of NeuN staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=5, CFD WT n=5, CFD Tau58 n=4, LD WT n=4, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G, DAPI-stained cell nuclei are in blue).

Expression of neurofibrillary tangles and hyperphosphorylated tau pathology in TAU58/2 mice fed with high-fat diets or normal chow diet

Intensity analysis of immunofluorescent staining for expression of neurofibrillary tangles (NFTs) using NF200 (Figure 8) in the hippocampal area did not show any significant differences among the three diet groups (NCD p=0.236, CFD p=0.06, LD p=0.509) or between the transgenic and wild-type mice of the same diet group (NCD vs CFD p=0.994, NCD vs LD p>0.999, CFD vs LD p=0.996).

Gallyas silver staining, which was performed to identify NFTs and NF-positive structures, revealed similar amounts of NFTs when normalised to hippocampal area among the three diet cohorts, with no difference observed between wild-type and transgenic mice (Figure 9). Positively stained cells (arrows shown in white, Figure 9 A) express silver iodide-bound neurofibrils under alkaline conditions[22,78,80]. Statistical comparisons were not done when n was less than 3, in either or both groups.

Tau phosphorylation in the hippocampus of TAU58/2 mice was analysed using immunofluorescent antibodies targeted to tau phosphorylated at Ser 396/404 (PHF1), Ser214 (pS214), and Ser422 (pS422). Analysis of immunofluorescent staining with the antibodies PHF1 (tau phosphorylated at Ser 396/404) (Figure 10) and tau phosphorylated at Ser422 (pS422) (Figure 11) did not show any significant difference among the diet cohorts. However, analysis of immunofluorescent staining intensities of tau phosphorylated at Ser214 (pS214) (Figure 12) revealed that the intensity of hyperphosphorylated tau in transgenic mice of each diet cohort was significantly high compared to the wild-type mice of the same cohort (p<0.001***). There was no significant difference among the transgenic mice between diet cohorts (Figure 12 G) (NCD vs CFD p=0.896, NCD vs LD p=0.991, CFD vs LD p=0.998).

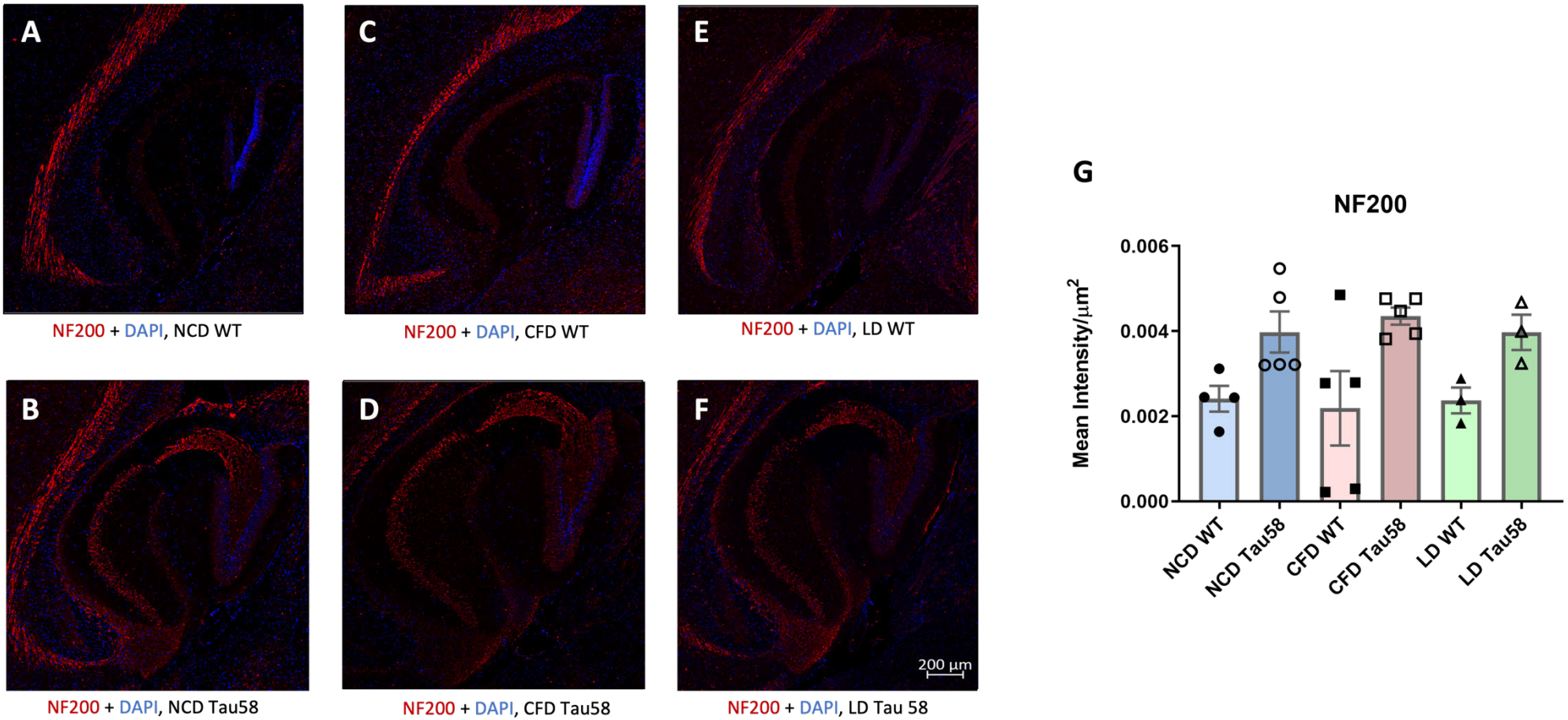

Figure 8: Representative images of neurofilaments (NF200) in the hippocampal area (A)Immunofluorescent staining of NF200 neurofibrillary tangles marker (red) in normal chow diet (NCD) wildtype (WT) mice, (B)Immunofluorescent staining of NF200 in NCD TAU58/2 (Tau58) mice, (C)Immunofluorescent staining of NF200 in coconut fat-derived (CFD) WT, (D)Immunofluorescent staining of NF200 in CFD Tau58, (E)Immunofluorescent staining of NF200 in lard diet (LD) WT, and (F)Immunofluorescent staining of NF200 in LD Tau58 (G) Mean intensity of NF200 staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=5, CFD WT n=5, CFD Tau58 n=5, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G).

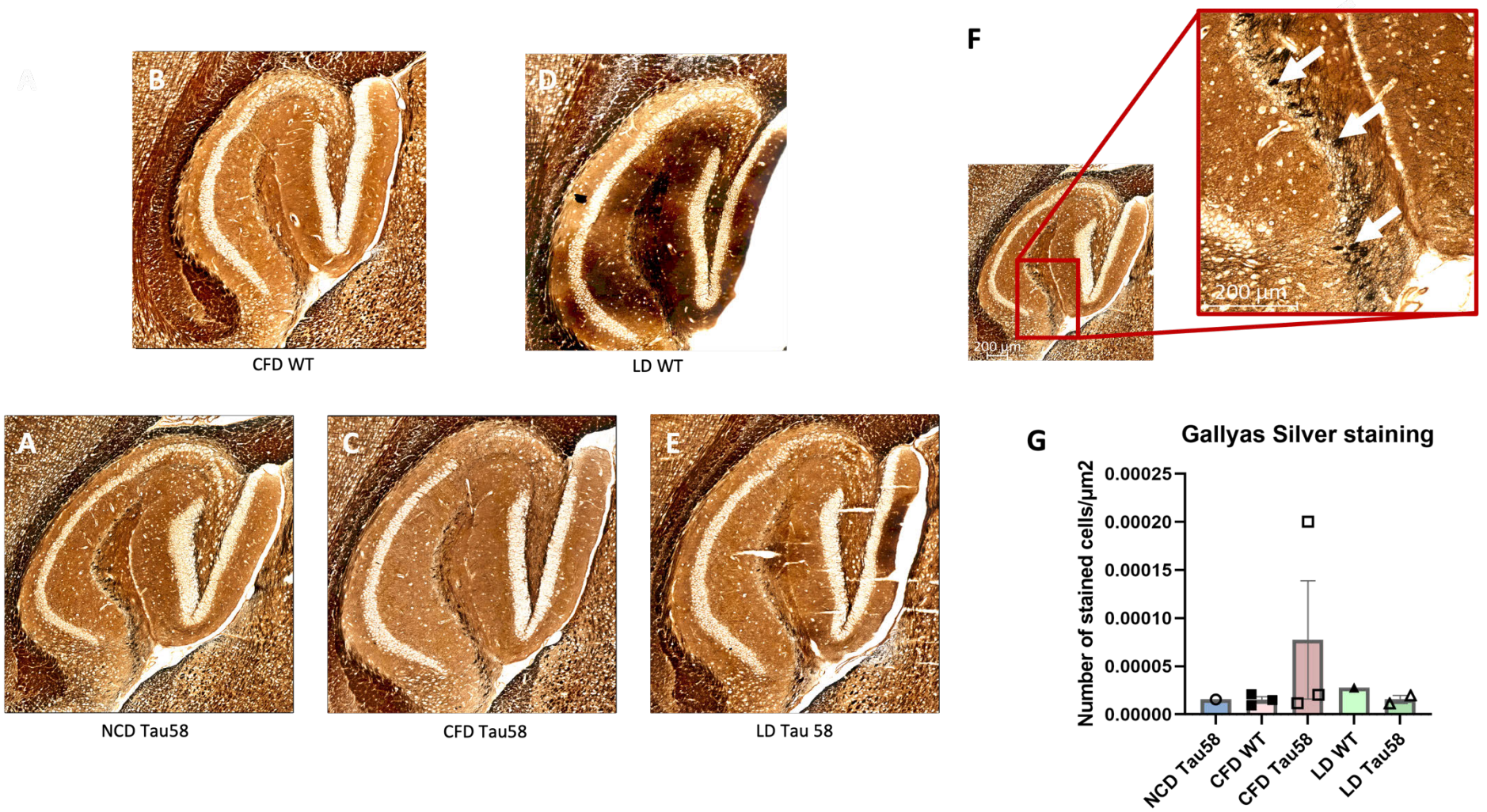

Figure 9: (A)-(E) Representative images of Gallyas silver staining of NFTs in the hippocampal area of wild-type (WT) and transgenic (Tau58) mice on normal chow diet (NCD), coconut-derived fat diet (CFD) or lard diet (LD) (NCD Tau58 n=1, CFD WT n=3, CFD Tau58 n=3, LD WT n=1, LD Tau58 n=2, data are shown as mean+SEM, individual data points are represented by the shapes in F, Scale bar= 200µm)).

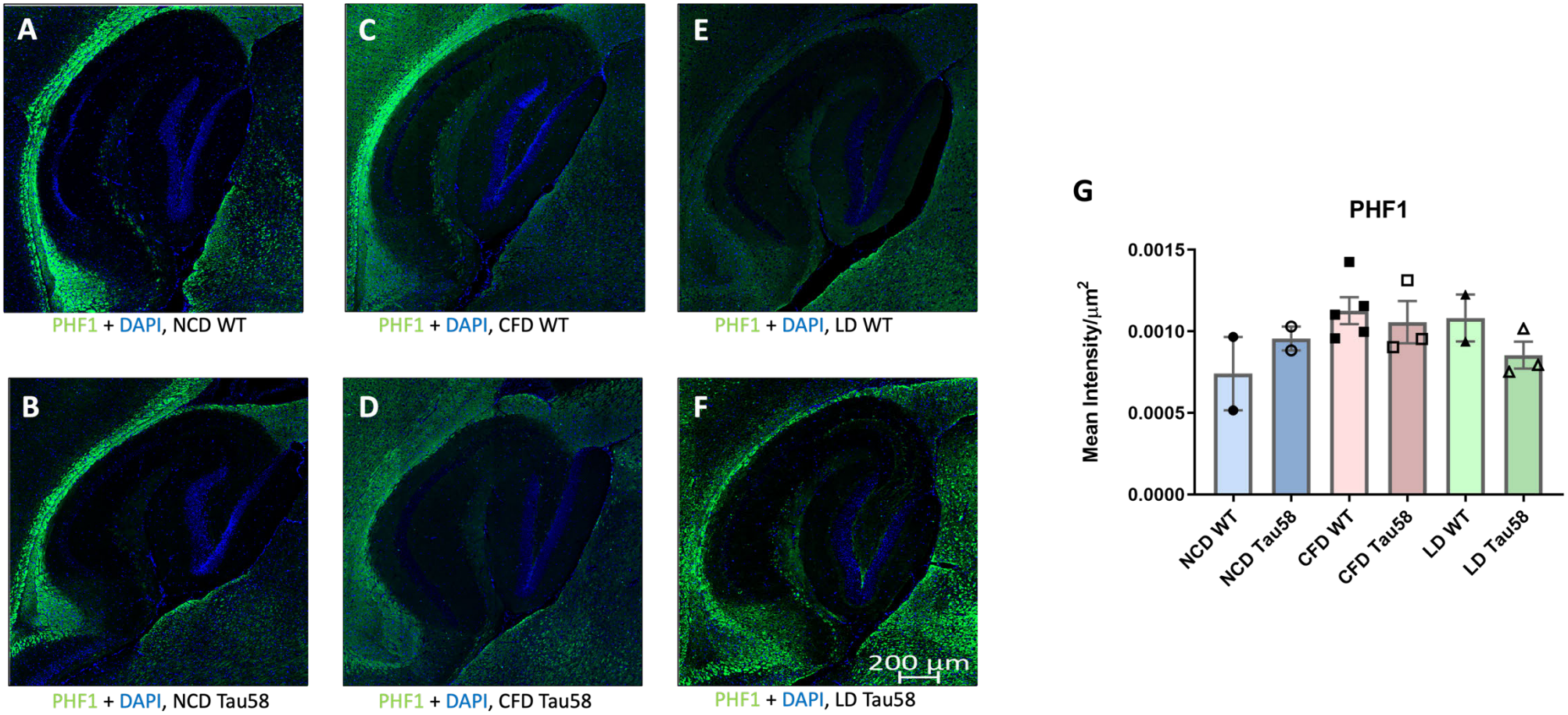

Figure 10: Representative images of hyperphosphorylated tau in the hippocampus of TAU58/2 mice identified by immunofluorescent staining of PHF1 (tau phos Ser 396/404) (green). (A)Immunofluorescent staining of PHF1 in normal chow diet (NCD) wildtype (WT) mice, (B) Immunofluorescent staining of PHF1 in NCD TAU58/2 (Tau58) mice, (C) Immunofluorescent staining of PHF1 in coconut fat-derived diet (CFD) WT, (D) Immunofluorescent staining of PHF1 in CFD Tau58, (E) Immunofluorescent staining of PHF1 in lard diet (LD) WT, and (F) Immunofluorescent staining of PHF1 in LD Tau58. (G) Mean intensity of PHF1 staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=5, CFD WT n=5, CFD Tau58 n=5, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G).

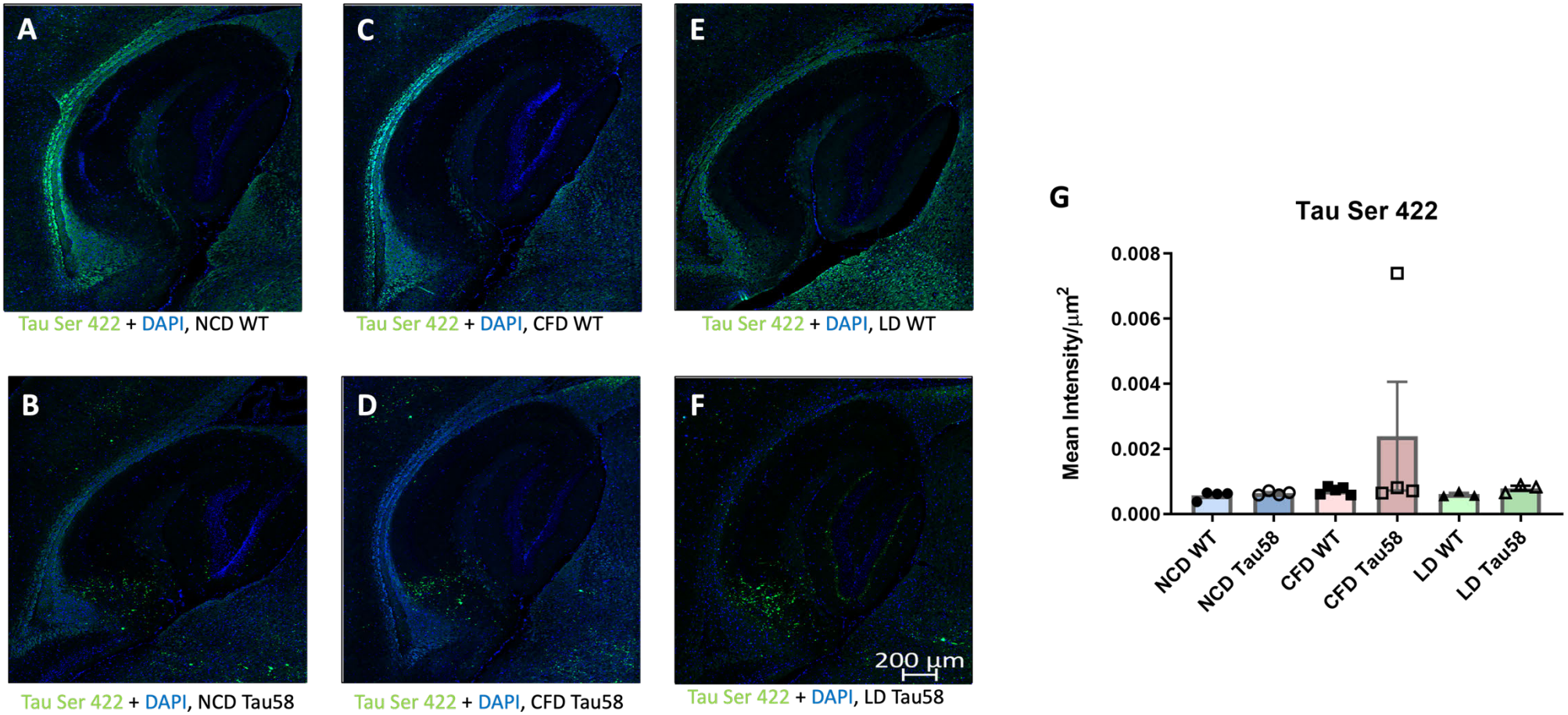

Figure 11: Representative images of hyperphosphorylated tau in the hippocampus identified by immunofluorescent staining of tau phos Ser422 (pS422, green) (A)Immunofluorescent staining of tau phos Ser422 in normal chow diet (NCD) wildtype (WT) mice, (B)Immunofluorescent staining of tau phos Ser422 in NCD TAU58/2 (Tau58) mice, (C)Immunofluorescent staining of tau phos Ser422 in coconut fat-derived (CFD) WT, (D)Immunofluorescent staining of tau phos Ser422 in CFD Tau58, (E)Immunofluorescent staining of tau phos Ser422 in lard diet (LD) WT, and (F)Immunofluorescent staining of tau phos Ser422 in LD Tau58. (G) Mean intensity of pS422 staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=4, CFD WT n=5, CFD Tau58 n=3, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G).

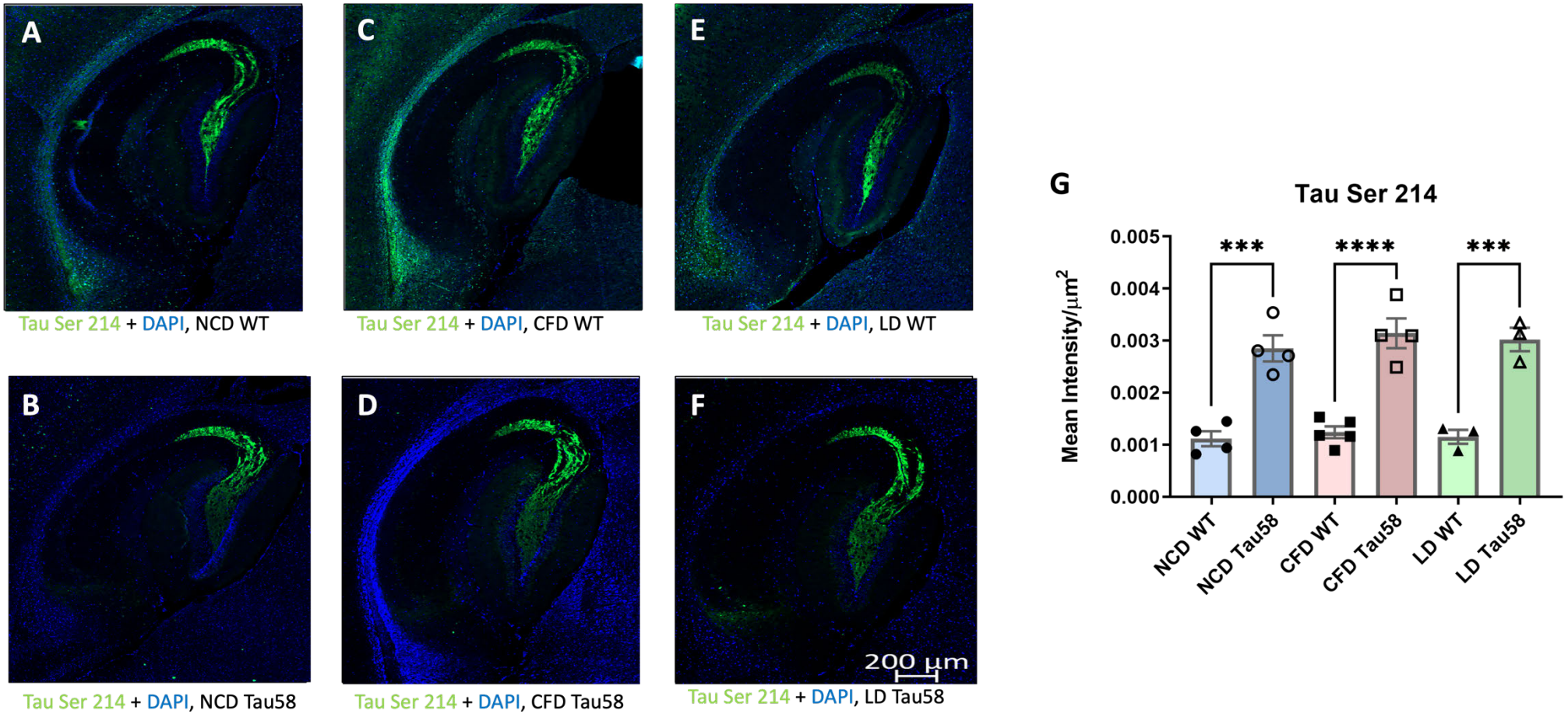

*Figure 12: Representative images of phosphorylated tau in the hippocampus (A)Immunofluorescent staining of tau phos Ser214 (pS214) in normal chow diet (NCD) WT, (B)Immunofluorescent staining of tau phos Ser214 in NCD Tau58, (C)Immunofluorescent staining of tau phos Ser214 in coconut fat derived (CFD) WT, (D)Immunofluorescent staining of tau phos Ser214 in CFD Tau58, (E)Immunofluorescent staining of tau phos Ser214 in lard diet (LD) WT, and (F)Immunofluorescent staining of tau phos Ser214 in LD Tau58. (NCD WT n=4, NCD Tau58 n=4, CFD WT n=5, CFD Tau58 n=4, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G). (G) Mean intensity of pS422 staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=4, CFD WT n=5, CFD Tau58 n=3, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G) ***=p<0.001, ****=p<0.0001.*

Microglia, and astrocytes expression in TAU58/2 mice fed with high fat diets and normal chow diet

Gliosis is a feature commonly observed in AD and FTD brains[22,86] . Therefore, to observe the effects that the three diets of NCD, CFD and LD may have on gliosis of microglia and astrocytes in the hippocampus of TAU58/2 mice, immunofluorescent staining of antibodies targeted to microglia (IBA1) and astrocytes (GFAP) was performed. Staining intensities of IBA1 (Figure 14) and GFAP (Figure 13) in the hippocampal area of TAU58/2 mice did not show a significant difference among the transgenic and wild-type mice of each diet cohort (IBA1: NCD $p=0.895$, CFD $p=0.859$, LD $p=0.994$) (GFAP: NCD $p=0.785$, CFD $p>0.999$) or among the diet cohorts themselves (IBA: NCD vs CFD $p=0.999$, NCD vs LD $p=0.976$, CFD vs LD $p=0.915$) (GDAP: NCD vs CFD $p=0.975$).

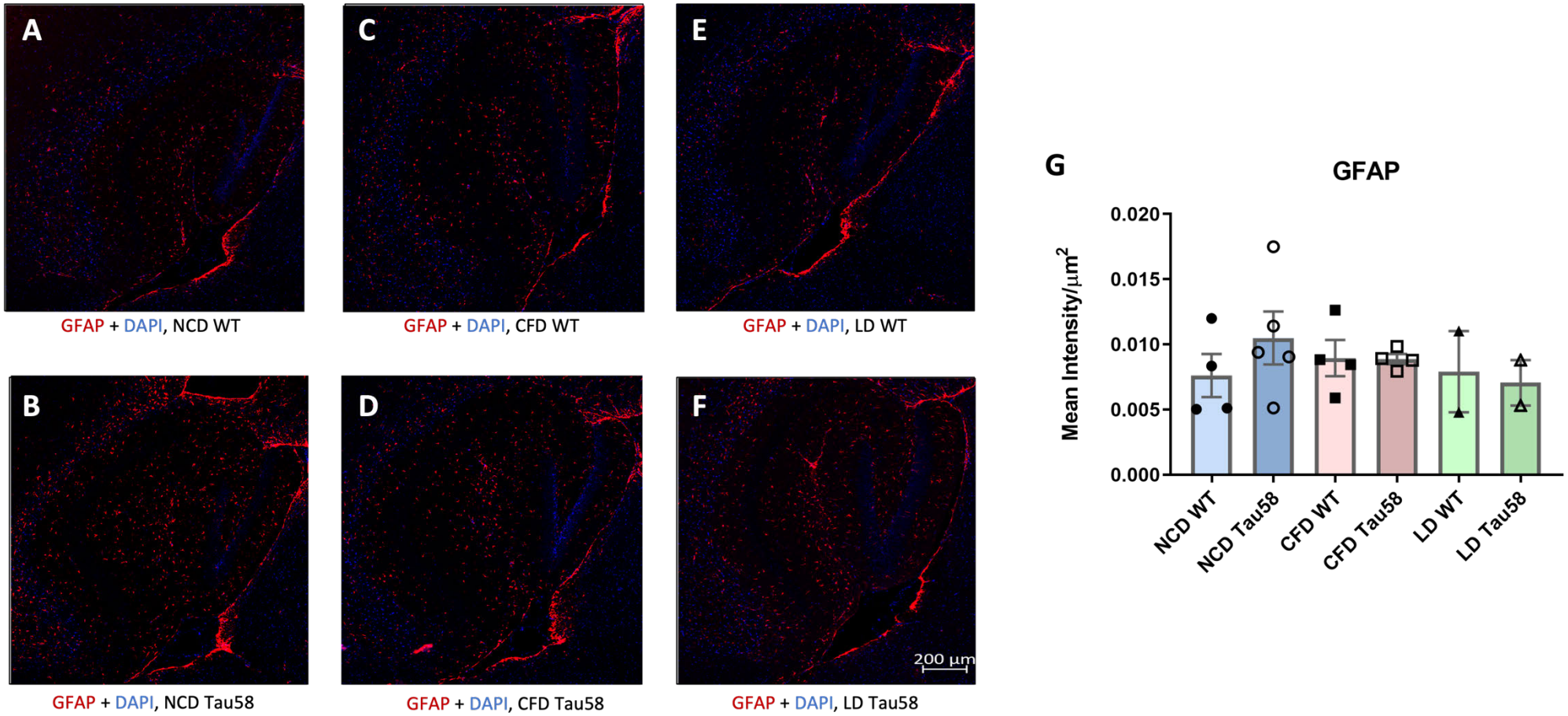

Figure 13: Representative images of distribution of astrocytes in the hippocampal area (A) Immunofluorescent staining of GFAP, astrocyte marker (red) in normal chow diet (NCD) wildtype (WT) mice, (B) Immunofluorescent staining of GFAP in NCD TAU58/2 (Tau58), (C) Immunofluorescent staining of GFAP in coconut fat derived (CFD) WT, (D) Immunofluorescent staining of NF200 in CFD Tau58, (E) Immunofluorescent staining of GFAP in lard diet (LD) WT, and (F) Immunofluorescent staining of GFAP in LD Tau58. (G) Mean intensity of GFAP staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=5, CFD WT n=4, CFD Tau58 n=4, LD WT n=2, LD Tau58 n=2, data are shown as mean+SEM, individual data points are represented by the shapes in G).

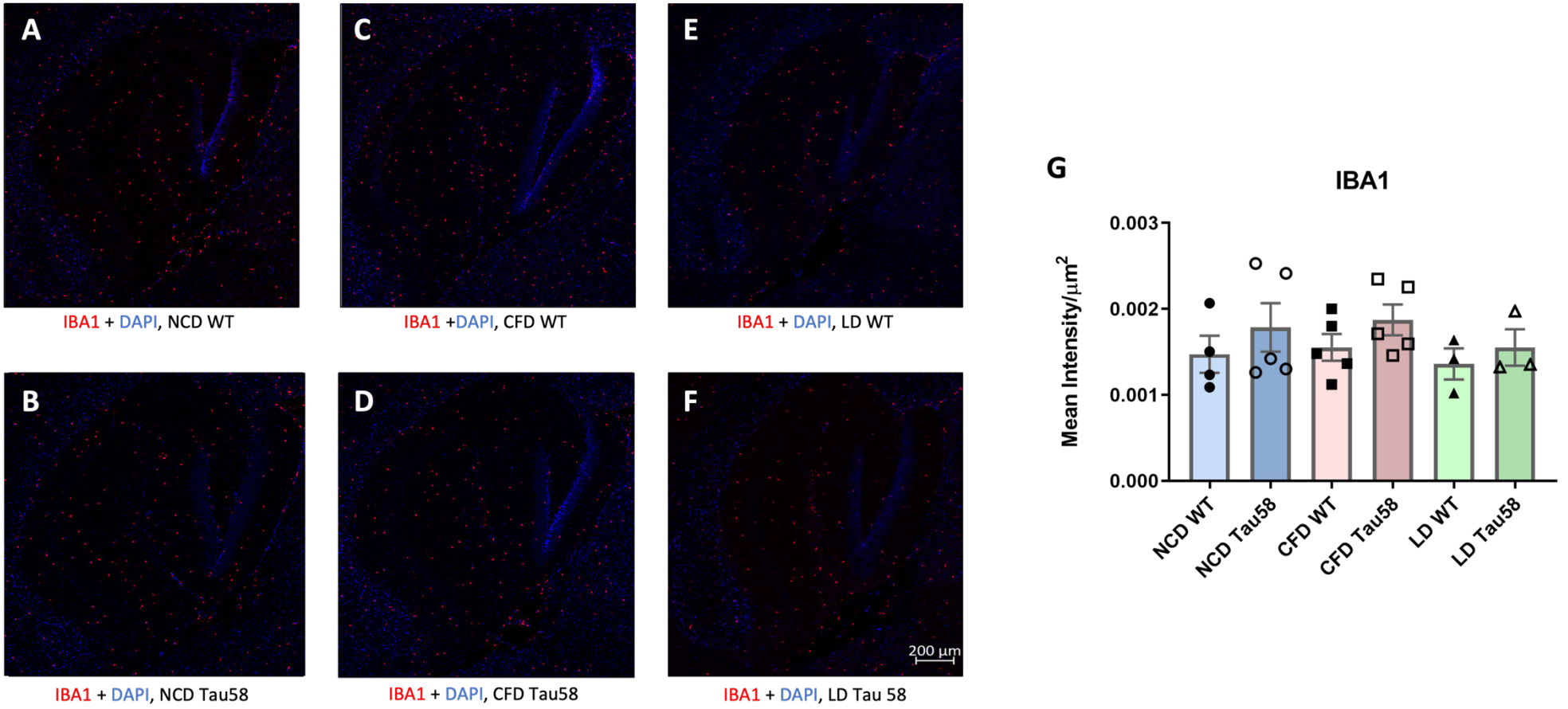

Figure 14: : Representative images of distribution of microglia in the hippocampal area (A) Immunofluorescent staining of IBA1, glial inflammatory marker (red) in normal chow diet (NCD) wildtype (WT) mice, (B) Immunofluorescent staining of IBA1 in NCD TAU58/2 (Tau58) mice, (C) Immunofluorescent staining of IBA1 in coconut fat-derived (CFD) WT, (D) Immunofluorescent staining of IBA1 in CFD Tau58, (E) Immunofluorescent staining of antibodies to IBA1 in lard diet (LD) WT, and (F) Immunofluorescent staining of IBA1 in LD Tau58.
(G) Mean intensity of IBA1 staining in the hippocampus normalised to area (NCD WT n=4, NCD Tau58 n=5, CFD WT n=5, CFD Tau58 n=5, LD WT n=3, LD Tau58 n=3, data are shown as mean+SEM, individual data points are represented by the shapes in G).

Chapter 5: Discussion

FTD and AD are progressive neurodegenerative diseases in which the disease burden increases with the patient's lifespan. Since there are no remedial, prophylactic, or preventative medicines available, there is enormous interest in finding alternative therapies that could have a positive impact on a patient's quality of life. With public and media interest, researchers have begun examining high fat and ketogenic diets with hopes of finding a therapeutic target for age-related neurodegenerative diseases such as FTD and AD[52]. Coconut oil, being a naturally occurring ketogenic substance that has also been shown to have antioxidant-rich and anti-inflammatory qualities, has taken the interest of many as a potential candidate in neurodegenerative disease research[49–51,87,88]. However, there are only a handful of published studies that specifically investigate any beneficial effects of coconut oil or its derivatives in AD, and studies on CO and FTD are virtually non-existent. The aims and approaches of this study were therefore focused on the effects of CO on both AD and FTD, and explicitly on tau (hyperphosphorylated) pathology which both diseases exhibit, and in which there seems to be a gap in extant knowledge.

MWM swim path analysis revealed that transgenic mice fed with coconut-derived fat performed significantly better than the mice on normal chow diet or lard diet in terms of the amount of hippocampal-dependent swim patterns. In addition, transgenic mice on the coconut fat-derived diet had the highest average swim path scores and the lowest average time taken to find the platform during the learning curve, performing better than wild-type mice, suggesting that this diet has a remarkable impact on hippocampal-dependent cognition. Although this enhanced learning did not translate into improvements in the probe trial, with no significant differences in the time spent in the platform quadrant, distance travelled to reach the platform quadrant, or the number of entries to the platform quadrant among the three diet cohorts (coconut fat diet, normal chow diet and lard diet), it does not detract from the earlier

observations of improvement in hippocampal-dependent swim patterns which relate to spatial learning and memory, as the probe trial is conducted to measure spatial bias and reference memory[85].

As expected, intensity analysis of immunofluorescent staining for human tau (Tau13) revealed that tau transgene expression was present in transgenic mice, with little staining observed in wild-type mice (which can be attributed to background intensity), validating the mouse model utilized for analysis of tau pathology. In addition, there was no significant difference in staining intensity of human tau among transgenic mice of the three diets, indicating that these diets did not influence the transgene expression. Intensity analysis of immunofluorescent staining for NFTs with NF200 showed a higher amount of staining in transgenic mice compared to wild-type mice, again providing further validation of the integrity of the TAU58/2 mouse model used given the transgene expression was not affected by the high fat diets. Also, in accordance with the above results, a highly significant difference was observed between the transgenic and wild type mice of the three diet groups in the expression of pathological tau phosphorylation at the early pathological marker site of Ser214 (pS214)[43], with transgenic mice showing a higher expression of phosphorylation. Tau phosphorylation at the late pathological marker sites of Ser422 (pS422) and Ser 396/404 (PHF1)[43] did not show a difference in expression between wildtype and transgenic mice at 5 months of age. Phosphorylation at Ser422 and Ser396/404 have previously been reported at 10 months of age, these findings show that hyperphosphorylation, at least at these sites, was not brought on earlier by either the coconut-derived or fat derived diets, as no difference was seen between transgenic mice on different diets. In the present study, no difference was observed between staining intensities of microglial (IBA1 marker) and astrocytic (GFAP marker) expression. Further discussion regarding this is outlined in detail later in the text.

In the current study, cognitive testing (MWM) demonstrated a significant improvement of hippocampal-dependent behaviour which indicate improvement of spatial learning and memory

in the group of mice fed with the diet consisting of coconut fat, while the histological staining intensity analysis did not show any significant differences between the three diet groups. This inconsistency between behavioural and histological analysis raises several points of discussion including. (1) Coconut oil may be altering amyloid beta plaque expression (and other pathologies) of AD and FTD, but not tau pathology; (2) Coconut oil may not be directly affecting tau hyperphosphorylation or deposition, but it may be affecting other aspects such as mitochondrial respiration, oxidative stress and inflammation in AD and FTD; (3) Coconut oil may be improving mental fatigue acting as a substitute fuel to glucose, but not memory and learning; (4) This study focused only on the hippocampus, yet AD and FTD are known to affect other regions of the brain including entorhinal and frontotemporal cortices; (5) Investigating different titrations of the coconut fat derived diet and/or individual compounds derived from CO in addition to pursuing combination diets could be more efficient in future follow up studies.

In the AD brain, increased Aβ peptides form Aβ amyloid fibrils develop into senile plaques, resulting in neurotoxicity that leads to neuronal cell death, and it is proposed that tau pathology may also be induced by Aβ senile plaque formation[89]. However, there is ongoing debate among researchers whether the main factor causing the progression of AD is in fact tau and not Aβ, and whether Aβ amyloidosis & tau pathology should be considered independent pathological events[89]. However, both tau pathology (hyperphosphorylated tau and NFTs) and Aβ pathology is observed in the neurodegenerative brain of AD patients[1,89]. FTD patient brains also show tau accumulation and hyperphosphorylation which ultimately lead to degeneration of neurons[6,90]. Several studies have been conducted on mouse models targeting Aβ pathology which have demonstrated neuroprotective outcomes of coconut oil and its derivatives[72,74,77,87,88,91]. Two studies have shown that Maltolyl p-coumarate, a compound found in coconut oil reduced cognitive deficits and apoptosis levels in Aβ42-injected rats and ferulic acid reduced cortical Aβ levels in an APP/PS1 (APPswe/PS1de9) AD transgenic mouse model[75,76]. Another study

on rats injected with Aβ placed on a diet of virgin coconut oil showed potential neuroprotective effects by normalising NLRP3 inflammasome levels, which is a critical component of innate immune system is activated in the presence of microbial infection and cellular damage[73]. A study conducted on rats with aluminium chloride-induced AD who were placed on a dietary formula fortified with virgin coconut oil also showed reduced cognitive deficits during behavioural tests, including Y maze, modified elevated plus maze and novel object recognition test and this study also showed improved brain architecture[77]. In addition, there are a few studies that show coconut oil to be beneficial in reducing TDP-43 pathology in ALS mouse models (ALS exists on a disease spectrum with FTD)[92,93]. Since the current study did not observe any pathological changes in the hippocampal area of TAU58/2 mice who were fed coconut fat-derived diet compared to mice who were fed normal chow and lard diets, it could suggest that coconut oil (CO) may be affecting Aβ pathology or TDP-43 pathology (in FTD), but not tau pathology. Therefore, in future studies it may be worthwhile replicating this study on mouse models recapitulating Aβ or TDP-43 pathology.

Ketogenic diets have been shown to increase mitochondrial respiration, support long-term neuronal potentiation, decrease oxidative stress and decrease inflammation[50,52,53]. CO is also well-known for its ketogenic, antioxidative, anti-inflammatory properties[51,52,55,71]. Therefore, considering that immunofluorescent staining did not show much of an effect of CO on hyperphosphorylated tau, however significant improvements in cognitive behaviour were observed with MWM testing, it suggests that CO may not be directly affecting tau hyperphosphorylation or deposition, instead, it may be improving areas such as mitochondrial respiration, and oxidative stress. There were no significant differences in staining intensities of microglia and astrocyte expression across the three diet cohorts. Microglia and astrocytes are the innate immune cells of the brain, coordinating the immune response, blood flow to brain through the blood brain barrier, metabolism and waste clearance within the brain[18,86,94]. In future, it would be beneficial to assess the number and morphology of microglia and astrocytes

across the three diets in other mouse models of Aβ pathology and TDP-43 pathology in the context of FTD. Given this study was a first pass analysis of diet and tau, histological analysis was limited to the investigation of the hippocampus, therefore, any peripheral changes with regards to mitochondrial respiration, oxidative stress and other key aspects of neuroinflammation such as alterations in activation states of these glial cells (e.g., activated state, resting state) were not assessed. Specifically, IBA1, being a pan microglial marker that labels all cells expressing microglial phenotype, may limit the ability to assess the inflammatory activation state or phenotype of microglial subtypes that exist within the brain in response to stimuli such as neurodegeneration[18]. GFAP, being a general astrocytic marker, may also limit the ability to assess the inflammatory activation state of various astrocytic subtypes[94]. In future potentially utilizing a panel of specific markers that label various microglial and astrocytic subtypes would be beneficial, given the activation state of glia may be an important factor in the context of neurodegeneration, however this was beyond the scope of the current study. Given the main focus of this study was to assess any of the potential effect different diets on hyperphosphorylated tau within the hippocampus, in future it would be beneficial to conduct further research that focuses on the effect of a coconut-derived high fat diet on mitochondrial respiration, oxidative stress and neuroinflammation in TAU58/2 mice utilising a panel of antibodies that target the expression of different glial and astrocytic activation states, and assess this expression in other areas of the brain (e.g. cortical) and peripheral organ systems as well such as skin, bladder, or intestines.

An energy disparity in the brain due to glucose hypometabolism and mitochondrial deficits is reported in neurodegeneration and in age-related neurodegenerative diseases such as AD and FTD. Ketones generated from the breakdown of medium-chain triglycerides act as substitute fuel to glucose[50,51,71]. Mental fatigue, which is a dimension of cognition[95], occurring from sustained cognitive activity, burns up glucose[95]. Thus, it is reasonable to propose that CO with its ketogenic ability may simply be improving the mental fatigue that occurs by the effortful

mental process of finding the platform in the MWM in Tau58/2 mice, however not necessarily be improving learning and memory. This study reflects this as shown by the discrepancy between the learning curve and probe trial findings. By the 5th day of trials, transgenic TAU58/2 mice on the coconut-derived fat diet (compared to the mice on normal chow and lard diets) took the least amount of time to find the platform. However, the probe trial which shows spatial bias and reference memory did not generate any changes among the three diet cohorts. Therefore, in future research it would be prudent to investigate metabolic effects using metabolic markers such as triglycerides and mitochondrial expression to address whether these aspects may be altered in TAU58/2 mice on such diets.

The hippocampus is thought to play an important role in storing long term memory, learning, spatial processing and navigation[96]. Other regions of the brain that are considered to be involved with memory are the amygdala, cerebellum, and the prefrontal cortex[97]. In addition, cortical areas such as the frontal lobe (body movement, intelligence, concentration, self-awareness, planning and behaviour), temporal lobe (memory, sequencing and organization), and parietal lobe (spatial and visual perception) are known to play roles of varying degrees when it comes to memory and learning[98]. The current study focused specifically on the hippocampus; therefore, it may be beneficial to focus on these other areas of the brain involved in memory and its associated pathways to determine how high fat diets affect tau phosphorylation and neurodegeneration within these regions. Furthermore, another important aspect to be considered is that FTD primarily affects the fronto-temporal regions of the brain, an area that was not assessed in the current study, which should be addressed in future studies.

Examining different titrations of the CFD and using individual compounds (medium chain fatty acids) derived from CO (caprylic acid, capric acid, and lauric acid, ferulic acid) in a similar replicated study may generate more promising results regarding diet and tau pathology. In addition, in studies such as this it is important to consider the timing and duration of the diets.

In the current study TAU58/2 mice were exclusively put on the three diets from weaning (postnatal day 21) to 3.5 months of age and cognitive deficits that may exhibit after this age were beyond the extent of the study.

Chapter 6: Conclusions and future directions

To conclude, MWM swim path analysis revealed that transgenic mice fed with the coconut fat diet displayed significantly higher hippocampal-dependent cognitive behaviour (swim patterns) when compared to normal chow and lard diets, suggesting that incorporation of coconut fat into diet may be beneficial in retaining learning and memory in AD and FTD. Furthermore, transgenic mice fed with the lard diet showed the least amount of hippocampal-dependent swim patterns compared to the wild-type mice fed with the same diet and compared to other diet cohorts, indicating that a diet of lard which consists of saturated fats (56-62%)[99], could in fact adversely affect cognition in AD and FTD. Immunofluorescent staining intensity analysis of human tau (Tau13) further validated the integrity of the TAU58/2 mouse model used within this study, with a significantly higher human tau expression in the transgenic mice compared to the wild-type mice, in addition to the finding that all three diets had no influence on the transgene expression demonstrated by no significant difference in the intensity of human tau among transgenic mice of the three diets. Although no difference between immunofluorescent staining intensities of antibodies against microglia (IBA1) and astrocytes (GFAP) was observed, this finding indicates there is a need to explore other avenues regarding neuroinflammation including the use of a panel of various inflammatory markers that can detect different activation states or label various morphological subtypes of glial cells, or label markers of other associated inflammatory pathways in the context of neurodegeneration in order to assess the effect coconut fat-derived diet may have on the neuroinflammatory states observed in AD and FTD.

Following the immunofluorescent staining intensity analysis, further quantification of results using Western blotting technique in brain tissue of Tau58/2 mice of three diet cohorts was intended. However, due to the challenges faced during the 2021 covid-19 lockdown, access to laboratory facilities was restricted and as such this analysis could not be completed. Therefore,

in future follow-up studies, western blot analysis should be performed to further validate the results on a molecular level.

The overarching aim of this study was to observe the effects of coconut oil on tau pathology in the neurodegenerative landscape of AD and FTD, given there has been no research that specifically explores this. While no significant histopathological changes in the post-mortem brain sections were observed, this study did demonstrate significant improvements in hippocampal-dependent cognition in TAU58/2 mice fed with coconut oil-derived fat. Collectively this study has uncovered a multitude of avenues to explore in the future in relation to coconut oil and tau pathology in neurodegeneration, to determine whether coconut oil could be considered an appropriate alternative treatment or used as a dietary supplement in AD and FTD.

As outlined, there is potential to explore unanswered questions arising from this study. The effects of coconut/ high-fat diets on mouse models of Aβ pathology specifically could be examined given there are limited published studies exploring control diets and coconut-fat diets exclusively and then comparing them to another high-fat diet such as the lard diet. Effects of coconut-derived fat on TDP-43 pathology in FTD could also be examined. Investigating the effects of these diets on mitochondrial respiration, oxidative stress, and neuroinflammation using different inflammatory markers of the central and peripheral nervous systems could also be beneficial. In addition, the study could be extended to investigating specific hippocampal regions (CA1, CA2, and CA3) to identify specific expression patterns at a higher magnification. The investigation of the effects of coconut oil on tau pathology in other brain regions that have known functions in learning and memory could also be pursued. Furthermore, manipulating different components of these diets such as the timing and duration, examining different titrations, and the effects that individual medium chain fatty acid constituents of coconut oil might have on AD and FTD could be considered.

Overall, data from this study demonstrates that while there is standardization and improvements to be made in basic laboratory research investigating the effect of diet on neurodegeneration before translating findings to human clinical trials, the significant improvement in cognitive testing demonstrated in this study indicates that a diet incorporating coconut fat could in fact be of benefit in ameliorating impairments of learning and memory in neurodegenerative tauopathies such as AD and FTD.

Chapter 7: References

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Diet

Meat Free Rat and Mouse Diet

A fixed formulation diet for Laboratory Rats and Mice fortified with vitamins and minerals to meet the requirements of breeding animals after the diet is autoclaved or irradiated.

- Minor modifications were made to this fixed formulation on 12 February 2014. Please contact us for details.
- All nutritional parameters of this diet meet or exceed the NRC guidelines for Rats and Mice.
- The diet has been designed as a general ration for breeding and early growth in all rat and mouse strains. The total fat content has been deliberately kept low at around 5%, to maximise the long term breeding performance of most strains.
- The formulation is designed to be fed ad-lib to rodents of all ages. There is some indication that growth performance in a minority of strains can be improved by increasing dietary energy (fat content). BalbC mice, DA rats and some of the modified strains appear to be most susceptible to this problem. Please contact us if you are concerned about this issue.
- Mammalian meals have been excluded from the diet, however the diet does contain fish meal. We have formulated totally vegetarian diets, and maintained colonies for some time on these diets. Please contact us if you require such a diet.
- The feed is manufactured in a cylindrical form with a diameter of around 12 mm, length is variable from 10 mm to 30 mm. We have found that this form is ideal for overhead hopper feeding, maximising the ease of handling whilst minimising fines formation and the risk of bridging in the feed hopper. Pellet strength has been kept lower than conventional pelletised diets. While this leads to a slight increase in transit and storage damage to the diet (fines generation), we have found that juvenile mice often have a lower feed intake on harder pellets.
- The diet is packed in permeable bags suitable for direct loading into an autoclave. It is recommended that the diet be autoclaved at 120$_0$ C for 20 minutes with a post autoclaving vacuum drying cycle. Some clumping of the diet can be expected, but the diet clumps can usually be easily broken. Modifying the drying time to leave some residual moisture in the diet can minimise the clumping. Do not autoclave at 135$_0$ C as this will result in significant clumping that will be difficult to break.

Calculated Nutritional Parameters as Fed

Protein	19.00%
Total Fat	4.60%
Crude Fibre	5.20%
Acid Detergent Fibre	7.70%
Neutral Detergent Fibre	15.50%
Total Carbohydrate	59.90%
Digestible Energy	14.2 MJ / Kg
% Total Calculated Energy From Protein	23.00%
% Total Calculated Energy From Lipids	12.00%

Ingredients

A Fixed formula ration using the following ingredients:
Wheat, barley, Lupins, Soya meal, Fish meal, Mixed vegetable oils, Canola oil, Salt, Calcium carbonate, Dicalcium phosphate, Magnesium oxide, and a Vitamin and trace mineral premix.

Added Vitamins as Fed

Vitamin A (Retinol)	10 000 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	100 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	100 mg/Kg
Vitamin B6 (Pryridoxine)	25 mg/Kg
Calcium Pantothenate	50 mg/Kg
Biotin	300 ug/Kg
Folic Acid	5.0 mg/Kg
Vitamin B12 (Cyancobalamin)	150 ug/Kg

Diet Form and Features

- Cereal grain base diet. 12 mm diameter pellets.
- Pack size 10 and 20 Kg Bags.
- Diet suitable for irradiation, also suitable for autoclave.
- Lead time 2 weeks

Added Trace Minerals as Fed

Magnesium	100 mg/Kg
Iron	70 mg/Kg
Copper	16 mg/Kg
Iodine	0.5 mg/Kg
Manganese	70 mg/Kg
Zinc	60 mg/Kg
Molybdenum	0.5 mg/Kg
Selenium	0.1 mg/Kg

Calculated Amino Acids as Fed

Valine	0.86%
Leucine	1.40%
Isoleucine	0.80%
Threonine	0.70%
Methionine	0.30%
Cysteine	0.30%
Lysine	0.90%
Phenylalanine	0.90%
Tyrosine	0.70%
Tryptophan	0.20%
Histidine	0.50%
Taurine	83.4 mg/Kg

Calculated Total Minerals as Fed	
Calcium	0.80%
Phosphorous	0.70%
Magnesium	0.20%
Sodium	0.20%
Potassium	0.80%
Sulphur	0.20%
Iron	200 mg/Kg
Copper	24 mg/Kg
Iodine	0.5 mg/Kg
Manganese	114 mg/Kg
Cobalt	0.6 mg/Kg
Zinc	90 mg/Kg
Molybdenum	1.2 mg/Kg
Selenium	0.4 mg/Kg
Cadmium	0.05 mg/Kg

Calculated Fatty Acid Composition as Fed	
Myristic Acid 14:0	0.03%
Palmitic Acid 16:0	0.54%
Stearic Acid 18:0	0.14%
Palmitoleic Acid 16:1	0.01%
Oleic Acid 18:1	1.96%
Gadoleic Acid 20:1	0.03%
Linoleic Acid 18:2 n6	1.41%
a Linolenic Acid 18:3 n3	0.31%
Arachadonic Acid 20:4 n6	0.01%
EPA 20:5 n3	0.02%
DHA 22:6 n3	0.05%
Total n3	0.38%
Total n6	1.42%
Total Mono Unsaturated Fats	2.06%
Total Polyunsaturated Fats	1.78%
Total Saturated Fats	0.76%

Calculated Total Vitamins as Fed	
Vitamin A (Retinol)	10 950 IU/Kg
Vitamin D (Cholecalciferol)	2 000 IU/Kg
Vitamin E (a Tocopherol acetate)	110 mg/Kg
Vitamin K (Menadione)	20 mg/Kg
Vitamin C (Ascorbic acid)	No data
Vitamin B1 (Thiamine)	80 mg/Kg
Vitamin B2 (Riboflavin)	30 mg/Kg
Niacin (Nicotinic acid)	145 mg/Kg
Vitamin B6 (Pryridoxine)	28 mg/Kg
Pantothenic Acid	60 mg/Kg
Biotin	410 ug/Kg
Folic Acid	5 mg/Kg
Inositol	No data
Vitamin B12 (Cyancobalamin)	150 ug/Kg
Choline	1 640 mg/Kg

Calculated data uses information from typical raw material composition. It could be expected that individual batches of diet will vary from this figure. **Diet post treatment by irradiation or auto clave could change these parameters**.

We are happy to provide full calculated nutritional information for all of our products, however we would like to emphasise that these diets have been specifically designed for manufacture by Specialty Feeds.

Product Data - D12331

Description
58 kcal% fat w/sucrose
Surwit Diet

Used in Research
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identi ed with the product name, description, lot number and expiration date.

Lead Time
IN-STOCK.
Ready for next day shipment.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.
Because of the high fat content is best if kept frozen.

Control Diets
D12329

Special Considerations
Contains no ber.
High in sodium.

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www.ResearchDiets.com

Formula

Product #D12331	gm%	kca %
Protein	23.0	16.4
Carbohydrate	35.5	25.5
Fat	35.8	58.0
Tota		100
kca /gm	5.56	

Ingredient	gm	kca
Casein, 30 Mesh	228	912
DL-Methionine	2	0
Maltodextrin 10	170	680
Corn Starch	0	0
Sucrose	175	700
Soybean Oil	25	225
Coconut Oil, Hydrogenated	333.5	3001.5
Mineral Mix S10001	40	0
Sodium Bicarbonate	10.5	0
Potassium Citrate, 1 H2O	4	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Red Dye #4	0.1	0
Total	1000.1	5558.5

Professor Richard Surwit designed these diets with us for his diet-induced obesity studies at Duke University.
Diets match 10/27/92 telephone speci cations of R. Surwit, Ph. D., Duke University. Formulated by E. A. Ulman, Ph.D., Research Diets, Inc. November 6, 1992.

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Report ▸ Repeat ▸ Revise

D12328, D12329, D12330 and D12331

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Product Data - D12492

Description
Rodent Diet with 60% kcal% fat.

Used in Research
Fatty Liver
In ammation
Obesity
Diabetes

Packaging
Product is packed in 12.5 kg box. Each box is identi ed with the product name, description, lot number and expiration date.

Lead Time
IN-STOCK.
Ready for next day shipment.

Gamma-Irradiation
Yes. Add 10 days to delivery time.

Form
Pellet, Powder, Liquid

Shelf Life
Most diets require storage in a cool dry environment. Stored correctly they should last 6 months.
Because of the high fat content is best if kept frozen.

Control Diets
D12450B, D12450J, D12450K

Formula

Product #D12492		gm%	kca %
Protein		26.2	20
Carbohydrate		26.3	20
Fat		34.9	60
	Tota		100
	kca /gm	5.24	

Ingredient	gm	kca
Casein, 30 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	245	2205
Mineral Mix S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0
Total	773.85	4057

Formulated by E. A. Ulman, Ph.D., Research Diets, Inc., 8/26/98 and 3/11/99.

*Typical analysis of cholesterol in lard = 0.72 mg/gram.
Cholesterol (mg)/4057 kcal = 216.4
Cholesterol (mg)/kg = 279.6

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D12450B, D12451, D12492 The "Or g na H gh Fat D ets"

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MACQUARIE University

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2018/019-42 **Date of Expiry:** 30 June 2022

Online Project ID: 0628

Full Approval Duration *19 July 2018 to 30 June 2022*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Professor Lars Ittner
Faculty of Medicine, Health and Human Sciences
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lars.ittner@mq.edu.au
[redacted]

Associate Investigators:
Annika van Hummel
Esmeralda Paric
Josefine Bertz
Troy Butler
Nicolle Morey
Jessica Spathos
Fabien Delerue
Yazi Ke
Thomas Fath
Janet van Eersel
Liming Hou
Magdalene Przybyla
[redacted]

Others Participating:
Yuanyuan Deng
Julia van der Hoven
Astrid Feiten
Carol Au
Daniel Tan
Holly Stefen
Gabbriella Chan
Stefan Guerra
Miheer Sabale
Tamara Tomanic
Holly Ahel
Andrea Chan
Emilija Robinson
Brittany Gilchrist
Fiona Bright
Mian Bi
Tim Karl
Fabian Kreilaus
Rossana Rosa Porto
Sian Genoud
Ole Tietz
Caitlin Finney
Victoria Marsters
Samantha Knott
SeongHee Jung
Sherilyn Yao
[redacted]

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or Manager, CAF: 9850 7780 / [redacted] and **Animal Welfare Officer**: 9850 7758 / [redacted]

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Dementia Research Centre – Neurodegeneration protocol: investigating disease mechanisms and development of new therapies**

Purpose: 5 - Research: Human or Animal Health and Welfare

Aims: to determine how neuropathological changes and genetic modifications in mouse models of neurodegenerative diseases affect different modes of brain functions and how to intervene in these processes with novel therapeutic approaches.

Surgical Procedures category: 5 - Major Surgery with Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Weight/Sex	Total	Supplier/Source
01 – Mouse	Any	Weaning to 24 months/Any/Any	99,970	ABR/ACR/DRC breeding and generation
01 – Mouse	Any	Embryos	1,560	ABR/ACR/DRC breeding and generation
			101,530	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

AEC Reference No.: 2018/019-42 **Date of Expiry:** 30 June 2022

Amendments approved by the AEC since initial approval:

1. **Amendment** – 10/12/2018 – Add Yazi Ke, Arne Ittner, Thomas Fath, Janet van Eersel, Liming Hou, Magdalene Przybyla, Yuanyuan Deng, Kristie Stefanoska, Julia van der Hoven, Astrid Feiten, Carol Au, Daniel Tan, Adam Martin and Emmanuel Prikas, Holly Stefen and Gabbriella Chan to project (Executive approved. Ratified by AEC 14 February 2019).
2. **Amendment –** 08/12/2018 – Add experimental procedure to include the use of foetal neurons (Executive approved. To be ratified by AEC 14 February 2019).
3. **Amendment –** 08/12/2018 – Additional animals requested (1560) (Executive approved. Ratified by AEC 14 February 2019).
4. **Amendment** – 12/02/2019 – Add Annika van Hummel, Esmeralda Paric, Josefine Bertz, Lucy da Silva, Troy Butler, Wei Lee, Prita Riana Asih, Nicolle Morey and Jessica Spathos to project (Executive approved. Ratified by AEC 14 March 2019).
5. **Amendment** – 23/02/2019 – Change of procedure to include newly approved SOPs (AEC approved 11 April 2019).
6. **Amendment** – 05/04/2019 – Remove Lucy Da Silva from project (Executive approved. Ratified by AEC 16 May 2019).
7. **Amendment** – 17/06/2019 – New experimental procedure (to further refine the brain delivery of AAV and peptides, in order to reduce doses used and off target effects) (AEC approved 18 July 2019).
8. **Amendment –** 18/07/2019 – Add Stefan Guerra to project (Executive approved. Ratified by AEC 15 August 2019).
9. **Amendment –** 18/07/2019 – Add Miheer Sabale to project (Executive approved. Ratified by AEC 15 August 2019).
10. **Amendment –** 01/03/2019 – Add Tamara Tomanic to project (Executive approved. Ratified by AEC 17 October 2019).
11. **Amendment –** 13/09/2019 – Additional procedure outlined in DRC SOP #58 (Approved by AEC 19 September 2019).
12. **Amendment –** 15/11/2019 – Add Holly Ahel to project (Executive approved. Ratified by AEC 12 December 2019).
13. **Amendment** – 24/01/2020 – Amend experimental procedure, technique and/or design (to include cell-based therapies as additional approach to their treatment program) (AEC approved 20 February 2020).
14. **Amendment** – 24/04/2020 - Add Amanda Ta to the project (Executive approved. Ratified by AEC 21 May 2020).
15. **Amendment** – 24/04/2020 - Add Andrea Chan to the project (Executive approved. Ratified by AEC 21 May 2020).
16. **Amendment** – 24/04/2020 - Add Emilija Robinson to the project (Executive approved. Ratified by AEC 21 May 2020).
17. **Amendment** – 24/04/2020 - Add Brittany Gilchrist to the project (Executive approved. Ratified by AEC 21 May 2020).
18. **Amendment** – 24/04/2020 – Remove Wei Lee from the project (Executive approved. Ratified by AEC 21 May 2020).
19. **Amendment –** 11/06/2020 - Add Fiona Bright as Associate Investigator (Executive approved. Ratified by AEC 23 July 2020).
20. **Amendment –** 29/06/2020 - Amend experimental procedure, technique and/or design (Approved by AEC 23/07/2020).
21. **Amendment** – 20/07/2020 – Amend experimental procedure, technique and/or design (Executive approved. Ratified by AEC 20 August 2020).
22. **Amendment** – 02/09/2020 – Amend experimental procedure, technique and/or design *(These experiments are in collaboration with on external company which has provided a refined method that we would like to apply for planned experiments).* (Executive approved. Ratified by AEC 15 October 2020).
23. **Amendment** – 03/10/2020 – Amend experimental procedure, technique and/or design *(Introduce a more complex refinement of the social interaction testing by testing the social preference in a dedicated arena).* (Executive approved. Ratified by AEC 15/10/2020).
24. **Amendment – 13/10/2020** - Add Mian Bi to project (Executive approved. Ratified by AEC 19/11/2020).
25. **Amendment – 13/10/2020** – Remove Neda Assareh from project (Executive approved. Ratified by AEC 19/11/2020).
26. **Amendment – 16/11/2020** – Additional experimental procedure/technique or design (Approved by AEC 17/12/2020).
27. **Amendment – 07/12/2020** – Add Tim Karl to project (Executive approved. Ratified by AEC 18/02/2021).
28. **Amendment – 07/12/2020** – Add Fabian Kreilaus to project (Executive approved. Ratified by AEC 18/02/2021).
29. **Amendment – 07/12/2020** – Add Rossana Rosa Porto to project (Executive approved. Ratified by AEC 18/02/2021).
30. **Amendment – 13/12/2020 –** Amend experimental procedure, technique and/or design (include a standardized mouse model of TBI (outlined in SOP DRC #62) (Approved by AEC 18/03/2021, subject to the conditions below).
31. **Amendment – 15/12/2020** – Amend experimental procedure, technique and/or design (*include time mating of mice with superovulation of breeding female).* (Executive approved. Ratified by AEC 18/02/2021).
32. **Amendment – 22/02/2021 –** Add Sian Genoud to project (Executive approved. Ratified by AEC 18/03/2021).
33. **Amendment – 22/02/2021 –** Add Ole Tietz to project (Executive approved. Ratified by AEC 18/03/2021).
34. **Amendment – 22/02/2021 –** Remove Amanda Tan from project (Executive approved. Ratified by AEC 18/03/2021).
35. **Amendment – 07/05/2021** – Add Caitlin Finney to project (Executive approved. Ratified by AEC 17/06/2021).
36. **Amendment – 07/05/2021** – Add Victoria Marsters to project (Executive approved. Ratified by AEC 17/06/2021).
37. **Amendment – 07/05/2021 –** Add Samantha Knott to project (Executive approved. Ratified by AEC 17/06/2021).
38. **Amendment – 07/05/2021 –** Add SeongHee Jung to project (Executive approved. Ratified by AEC 17/06/2021).
39. **Amendment – 18/06/2021** – Remove Arne Ittner, Kristie Stefanoska, Prita Asih and Emmanuel Prikas from project (Approved by AEC 17/06/2021).
40. **Amendment – 07/07/2021** – Additional Experimental procedure, technique and/or design (refinement of delivery of substances – removing a hazard). (Executive approved. Ratified by AEC 19 August 2021).
41. **Amendment – 15/10/2021** – Add Sherilyn Yao to project (Executive approved. Ratified by AEC 18 November 2021).

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers' licence.

Conditions of Approval:

Amendment – 13/12/2020 – 1. It is mandatory that the AWO or delegate observe the first surgery (or more than one surgery, at their discretion), 2. A local anaesthetic must be supplied subcutaneously prior to the incision being made over the skull; 3. The AWO or delegate/s invited to attend to post-op monitoring for the first days after surgery.

Progress Report – 13/07/2021 – Approved for one month. A progress report is to be submitted for review at the 19 August 2021 AEC meeting, providing justification in the vast difference in the numbers.

Dr Angela Laird (Chair, Animal Ethics Committee) **Approval Date: 18 November 2021**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

MACQUARIE University

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2017/053-32 **Date of Expiry:** 31 December 2022

Online Project ID: 0336

Full Approval Duration: *01 January 2018 to 31 December 2022*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
Professor Lars Ittner
Faculty of Medicine, Health and Human Sciences
Macquarie University NSW 2109
lars.ittner@mq.edu.au
[redacted]

Others Participating:
Fabien Delerue
Nicolle Morey
Jessica Spathos
Troy Butler
Annika van Hummel
Magdalena Przybyla
Tamara Tomanic
Yazi Ke
Astrid Feiten
Carol Au
Daniel Tan
Esmeralda Paric
Holly Stefen
[redacted]

Others Participating:
Janet van Eersel
Josefine Bertz
Julia van der Hoven
Liming Hou
Yuanyuan Deng
Gabriella Chan
Stefan Guerra
Miheer Sabale
Andrea Chan
Emilija Robinson
Holly Ahel
Brittany Gilchrist
Fiona Bright
Mian Bi
Ole Tietz
Caitlin Finney
Victoria Marsters
Sian Genoud
Samantha Knott
SeongHee Jung
Sherilyn Yao
[redacted]

In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
~~or Manager, CAF: 9850 7780 /~~ [redacted] and ~~Animal Welfare Officer: 9850 7758 /~~ [redacted]

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: **Breeding and Maintenance of Dementia Research Centre Mouse Colony**

Purpose: 5 - Research: Human or Animal Health and Welfare

Aims:
1. To transfer mice from the current breeding colony of the Dementia Research Unit at the University of New South Wales to the MARS Central Animal Facility to establish these lines for the new Dementia Research Centre at Macquarie University
2. To expand the colonies and age mice for subsequent studies of the new Dementia Research Centre at Macquarie University
3. To collect tissues from mice that have reached specific ages for biochemical and histological analysis.

Surgical Procedures category: 2 - Animal Unconscious without Recovery

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Weight/Sex	Total	Supplier/Source
01 – Mouse	As per Attachment A of Application	Any	46,560	Dementia Research Unit Colony, University of NSW
			46,560	

Location of research:

Location	*Full street address*
Central Animal Facility	Building F9A, Research Park Drive, Macquarie University, NSW 2109

Amendments approved by the AEC since initial approval:

1. **Amendment** - **28/08/2018** - Add Nicolle Morey and Jessica Spathos to protocol (Executive approved. Ratified by AEC 18 October 2018).
2. **Amendment** – **17/09/2018** - Add Troy Butler and Dr Annika van Hummel to protocol (Executive approved. Ratified by AEC 18 October 2018).

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

3. **Amendment** – **11/02/2019** – Add Magdalena Przybyla, Annika Van Hummel, Arne Ittner, Astrid Feiten, Carol Au, Danial Tan, Emmanuel Prikas, Esmerelda Paric, Holly Stefen, Janet van Eersel, Josefine Bertz, Julia van der Hoven, Kristie Stefanoska, Liming Hou, Lucy da Silva, Troy Butler, Wei Lee, Yuanyuan Deng, Prita Riana Asih, Nicolle Morey and Jessica Spathos to protocol (Executive approved. To be ratified by AEC 14 March 2019).
4. **Amendment** – **05/04/2019** – Remove Lucy Da Silva from project (Executive approved. Ratified by AEC 16 May 2019).
5. **Amendment** – **04/07/2019** – Add Gabriella Chan and Neda Assareh to project (Executive approved. Ratified by AEC 15 August 2019).
6. **Amendment** – **18/07/2019** – Add Stefan Guerra and Miheer Sabale to project (Executive approved. Ratified by AEC 15 August 2019).
7. **Amendment** – **02/08/2019** – Add Yazi Ke and Tamara Tomanic to project (Executive approved. Ratified by AEC 19 September 2019).
8. **Amendments** – **24/01/2020** – Add Amanda Tan, Andrea Chan and Emilija Robinson to project (Executive approved. Ratified by AEC 20 February 2020).
9. **Amendment – 24/04/2020** – Add Holly Ahel to the protocol (Executive approved. Ratified by AEC 21 May 2020).
10. **Amendment – 24/04/2020** – Add Brittany Gilchrist to the protocol (Executive approved. Ratified by AEC 21 May 2020).
11. **Amendment – 24/04/2020** – Remove Wei Lee from the protocol (Executive approved. Ratified by AEC 21 May 2020).
12. **Amendment – 11/06/2020** - Add Fiona Bright as Associate Investigator (Executive approved. Ratified by AEC 23 July 2020).
13. **Amendment – 13/10/2020** – Add Mian Bi to project (Executive approved. Ratified by AEC 19/11/2020).
14. **Amendment – 13/10/2020** – Remove Neda Assareh from project (Executive approved. Ratified by AEC 19/11/2020).
15. **Amendment – 23/11/2020** – Addition of a shipment of mice to Flinders University in Adelaide, South Australia (Executive approved. Ratified by AEC 17 December 2020).
16. **Amendment – 22/02/2021** – Add Ole Tietz to project (Executive approved. Ratified by AEC 18/03/2021).
17. **Amendment – 22/02/2021** – Remove Amanda Tan from project (Executive approved. Ratified by AEC 18/03/2021).
18. **Amendment – 07/05/2021** – Add Caitlin Finney to project (Executive approved. Ratified by AEC 17/06/2021).
19. **Amendment – 07/05/2021** – Add Victoria Marsters to project (Executive approved. Ratified by AEC 17/06/2021).
20. **Amendment – 07/05/2021** – Add Sian Genoud to project (Executive approved. Ratified by AEC 17/06/2021).
21. **Amendment – 07/05/2021 –** Add Samantha Knott to project (Executive approved. Ratified by AEC 17/06/2021).
22. **Amendment – 07/05/2021 –** Add SeongHee Jung to project (Executive approved. Ratified by AEC 17/06/2021).
23. **Amendment – 18/06/2021** – Remove Arne Ittner, Kristie Stefanoska, Prita Asih and Emmanuel Prikas from protocol (Approved by AEC 17/06/2021).
24. **Amendment – 15/10/2021 –** Add Sherilyn Yao to project (Executive approved. Ratified by AEC 18 November 2021).

Conditions of Approval:

1. **Amendment – 23/11/2020** – Mice cannot be moved until a valid ARA from Flinders University has been reviewed and final clearance is granted by the Animal Welfare Officer, Macquarie University.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers licence.

Dr Angela Laird (Chair, Animal Ethics Committee) **Approval Date: 18 November 2021**

Adapted from Form C (issued under part IV of the Animal Research Act, 1985)

03/01/2018

Dear Lars Ittner,

Re:"Breeding and analysis of genetically modified mice " 0101- **520170101201**

NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective 03/01/2018.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Work requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified

Please note the following standard requirements of approval:

1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

Reporting: Annual progress reports are required for this project and a **Final Report for this project will be due on: 03/01/2023**

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this letter as this is your formal notification of final Biosafety approval. Also a copy of record submitted by Macquarie University to the OGTR.

Kind Regards,

Associate Professor Subramanyam Vemulpad

Institutional Biosafety Committee Chair,
Macquarie University Research Office
Level 3, Research Hub, Building C5C East
Macquarie University, NSW 2109 Australia
T: +61 2 9850 4063
E: biosafety@mq.edu.au
W: mq.edu.au/research

21/06/2018

Dear Mr Lars Ittner,

Re:"Gene modification – studies into the physiology and pathology of the nervous system " Project ID 0335

NOTIFICATION OF A NOTIFIABLE LOW RISK DEALING (NLRD)

The above application has been reviewed by the Institutional Biosafety Committee (IBC) and has been approved as an NLRD, effective 21/06/2018.

This approval is subject to the following standard conditions:

- The NLRD is conducted by persons with appropriate training and experience, within a facility certified to either Physical Containment level 1 (PC1) or PC2.
- Work requiring Quarantine Containment level 2 (QC2) does not commence until the facility has been certified

Please note the following standard requirements of approval:

1. Approval will be for a period of 5 years subject to the provision of annual reports. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. Please contact the Committee Secretary at biosafety@mq.edu.au for a copy of the annual report.

Reporting: Annual progress reports are required for this project and a **Final Report for this project will be due on: 21/06/2023**

2. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of approval to an external organisation as evidence that you have approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this letter as this is your formal notification of final Biosafety approval. Also a copy of record submitted by Macquarie University to the OGTR.

Kind Regards,

Associate Professor Subramanyam Vemulpad

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